ZEBRAFISH REVEAL VARIABLE EFFECTS OF *ESCHERICHIA COLI* DURING SEPSIS

by

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A dissertation submitted to the faculty of
The University of Utah
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

in

Microbiology and Immunology

Department of Pathology

The University of Utah

August 2016

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The University of Utah Graduate School

STATEMENT OF DISSERTATION APPROVAL

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ABSTRACT

Sepsis is a life-threatening systemic inflammatory condition that is characterized by a high degree of patient heterogeneity, making it notoriously difficult to diagnose and treat. Among the most common and lethal causes of sepsis are strains of Extraintestinal Pathogenic *Escherichia coli* (ExPEC). These genetically diverse pathogens are becoming increasingly problematic due to the rise of multidrug resistant strains. During sepsis, the infecting microbes are commonly viewed as generic inducers of inflammation, while the host background is considered the primary variable affecting disease progression and outcome. My doctoral research challenges this assumption and establishes a novel zebrafish embryo infection model to study the effects of ExPEC strain differences on the maladaptive immune responses that are induced during sepsis.

Zebrafish embryos infected with ExPEC isolates display many of the key pathophysiological features seen in human septic patients, including dysregulated inflammatory responses (cytokine storms), tachycardia, and endothelial leakage. Mirroring what is seen in human patients, antibiotic therapy reduces bacterial titers in infected embryos and improves host survival rates, but is only effective within limited time frames, and surviving animals often develop

lasting edema and other defects. Intriguingly, genetically distant ExPEC isolates stimulate markedly different host responses, including disparate levels of inflammatory (e.g., IL-1 β, TNF-α) and immunomodulatory (e.g., IL-10) mediators. These variances are attributable to differential activation of TLR5 by these strains, which vary in the levels and the serotypes of flagellin that they express. To examine the specific effects of IL-10 on disease progression, I created and begun to characterize engineered gene knockout and inducible transgenic zebrafish lines for this cytokine. Altogether, my graduate research establishes zebrafish as a relevant model for studying sepsis and highlights the ability of genetically distinct ExPEC isolates to induce divergent host responses independent of baseline host attributes and implicates bacterial flagellin as a key mediator of inflammation during sepsis.

In memory of James Barber.

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LIST OF ABBREVIATIONS

BEC..... Blood *Escherichia coli*
CFU.....Colony forming units
DIC..... Disseminated intravascular coagulation
EGFP .....Enhanced green fluorescent protein
ExPEC ..... Extraintestinal pathogen *Escherichia coli*
FliC..... Flagellin
GFP..... Green fluorescent protein
GO ..... Gene ontology
hpf.....Hours post-fertilization
hpi .....Hours post-inoculation
HRMA .....High resolution melt analysis
IL-1β..... Interleukin 1β
IL-10..... Interleukin 10
kDA .....Kilodalton
kb .....Kilobase
KEGG.....Kyoto encyclopedia of genes and genomes
LPS .....Lipopolysaccharide
MAP ..... Mitogen-activated protein
MDR..... Multidrug resistant
MLST .....Multilocus sequence typing
MO .....Morpholino oligonucleotide
mRNA .....Messenger ribonucleic acid
NFκB..... Nuclear factor kappa-light-chain-enhances of activated B cells
NMEC ..... Neonatal meningitis *Escherichia coli*
PBS.....Phosphate buffered saline
qRT-PCR .....Quantitative reverse transcription polymerase chain reaction
TALEN .....Transcription Activator-Like Effector Nuclease
TLR ..... Toll-like receptor

ACKNOWLEDGEMENTS

I am greatly indebted to my PhD mentor, Dr. Matthew Mulvey, for his continual support, devotion to research, and genuine interest in everything from bacteria to books. I am also appreciative of the freedom he gave me to develop my thesis project(s) into what they are today. Without him and the rigorous scientific environment he has created in his lab, I would not have developed into the scientist I am today. I am also thankful to the crew of Mulvey lab members past and present for their scientific insight and helpful advice.

I would also like to acknowledge my thesis committee members of Dr. David Grunwald, Dr. June Round, Dr. Janis Weis, and Dr. Guy Zimmerman for their guidance and support throughout my graduate career. I am also grateful to Janis and the Microbial Pathogenesis Training Grant for funding and providing myriad opportunities to immerse myself further into the world of infection biology.

On a more personal note, I would like to recognize my mom for being my biggest supporter and the most inspiring person I know. I am forever indebted to her for the sacrifices she has made to get me to where I am today. I am also grateful to my husband, Matt, and our two furry children for all the love, laughter and support provided by them. They make my life better. And finally, I am

thankful to all of my friends and to the mountains of Utah for all the adventures they gave me; without these I would not have survived graduate school.

CHAPTER 1

INTRODUCTION

Escherichia coli: a Diverse Opportunist

Escherichia coli is an incredibly diverse organism both genetically and phenotypically. It is a Gram-negative rod-shaped organism commonly found in the lower intestines of warm-blooded animals, but can also be found environmentally in water and soil. Within the intestinal tract, it is often viewed as a benign commensal that provides the host with essential vitamins and aids in the digestion of metabolites. However, there are strains of enteropathogenic *E. coli* that are capable of causing lethal diarrheal disease. *E. coli* isolates causing disease outside of the gastrointestinal tract, termed Extraintestinal Pathogenic *Escherichia coli* (ExPEC), are also very problematic causing a diverse range of conditions including meningitis, sepsis, and urinary tract infections (UTIs) [1]. UTIs are the one of the most common bacterial infections, with an estimated 8 million UTIs in the United States each year and 50% of women experiencing at least one during their lifetime [2]. ExPEC are the predominant organism responsible for UTIs, representing >80% of these infections. *E. coli* is also a leading cause of sepsis. The role of this organism during sepsis will be discussed in greater detail later in this chapter as well as in Chapters 2 and 3.

The genomes of ExPEC strains are comprised of a set of core genes common to nonpathogenic and pathogenic *E. coli* strains, but also contain virulence factors such as iron acquisition systems, capsular antigens, adhesins, and secreted toxins. Strikingly, gene content between individual clinical ExPEC strains can differ by as much as 30% and the pan genome, or total number of

distinct genes among all ExPEC isolates is estimated to be upwards of 14,000 [3-5]. This diversity has evolved likely as a means to allow ExPEC strains to survive and replicate under myriad environmental conditions and stresses. However, this genetic variation also presents significant challenges for the diagnosis and treatment of *E. coli*-initiated disease and no single gene or collection of genes has been identified as a universal indicator of pathogenesis or responsiveness to treatment.

Sepsis and the Role of *E. coli* in Disease

Sepsis is the overwhelming maladaptive activation of the immune system in response to invading microorganisms. More than 1 million individuals are admitted to the hospital with sepsis every year in the USA. With a mortality rate of 25-50% it kills more people in the US annually than prostate cancer, breast cancer and AIDS combined [6, 7]. Hospitalization rates for sepsis are also on the rise and have more than doubled since 2000 [7]. In addition to its unacceptably high mortality rate, sepsis places a significant financial burden on the healthcare system, being the most expensive cause for hospitalization and costing the healthcare system over $20 billion dollars per year in total [8].

While anyone can get sepsis, immunocomprimised populations such as the elderly, neonates and infants, AIDS patients, and people undergoing chemotherapy are particularly vulnerable. Following the widespread activation of pathogen recognition receptors and the corresponding increase in immune signal transduction cascades, a “cytokine storm” of inflammatory mediators is released,

leading to disseminated intravascular coagulation, endothelial leakage, pulmonary and systemic edema, and organ damage.

One of the key issues concerning sepsis is the heterogeneity seen in the patient population, making diagnosis and treatment especially difficult. Differences in host background are often viewed as the root cause of this, but the contribution of bacteria even within the same species remains largely unexplored. On account of this heterogeneity there are currently no satisfactory biomarkers for the diagnosis of sepsis or specific antisepsis therapies, so patient management relies on early administration of antibiotics and resuscitation with intravenous fluids and vasoactive drugs when needed. Antibiotic therapy should be initiated within 1 hour of a sepsis diagnosis. Each hour this is delayed is associated with a 7% increase in mortality [9]. Finally, those lucky enough to survive sepsis are often left with long term complications such as cognitive impairment and organ dysfunction and suffer a high rate of hospital readmission [10-13].

E. coli is the leading cause of bacteremia and the second most common cause of sepsis, with only Group B *Streptococcus* and *Staphylococcus aureus* being more prevalent in neonates and adults, respectively [14-18]. Strikingly *E. coli* is generally more inflammatory and more likely to cause death during sepsis relative to Gram-positive pathogens and other Gram-negative bacteria [19, 20]. Adding to the problem is the ongoing rise of multidrug resistant *E. coli* strains that are encroaching on the ability to effectively treat the underlying infection during

sepsis [21, 22]. Given the functional redundancy in virulence factors utilized by ExPEC strains to deal with host defenses and the challenges of a systemic infection, epidemiological and genomics-based studies have failed to identify a single set of bacterial genes that consistently correlates with the survival or lethality of ExPEC strains within the bloodstream of the human host [3, 23-28].

Despite a large volume of research on sepsis (at the time of writing there are greater than 150,000 results on PubMed for a search of “sepsis OR septicemia”), no novel drugs have been approved for the treatment of sepsis. This has been attributed partially to difficulties in moving findings from basic research into clinical trials and has brought up questions regarding the validity of many commonly used sepsis models [29, 30]. The most basic of sepsis models is simply the *in vitro* addition of bacteria or microbial products (i.e. LPS) to whole blood, endothelial cell lines, or innate immune cell lines [31-33]. Nevertheless, the predominant model system for the experimental study of sepsis is the mouse, where sepsis is initiated through the administration of LPS, viable pathogens, or cecal ligation and puncture (CLP) where the cecum is surgically punctured, releasing fecal material into the peritoneal cavity. Models involving LPS challenge of young mice in particular have come under fire in recent years, as it is becoming increasingly clear that the complex pathophysiological mechanisms of sepsis are not being modeled under these conditions [30, 34]. CLP has historically been the gold standard for the study of sepsis, but it is labor intensive and also reliant on the enteric microbiota of the mouse, which can vary between

animal facilities and differing environmental factors. In Chapter 2, I introduce zebrafish embryos as a novel, high throughput model system for the study of sepsis that recapitulates many key physiological hallmarks of disease. Furthermore, in Chapter 2 I demonstrate that genetically distinct *E. coli* strains are capable of causing markedly diverse host responses during sepsis, offering a partial explanation for the heterogeneity seen during sepsis. In Chapter 3 I explore the contributions of bacterial diversity to host response further and go on to demonstrate that for two ExPEC strains, these differences are attributable to variable levels of flagellin expression and differences in the ability of discrete flagellin variants to stimulate the host receptor TLR5.

Zebrafish as a Model for Host-Microbe Interactions

Early zebrafish research focused on embryonic development. However given its compelling advantages, it is now emerging as a valuable model for the study of host-microbe interactions, inflammation, and the immune system. Zebrafish are hardy, easy to maintain, and feature external development, optical transparency during early life, forward and reverse genetics, and vertebrate physiology. Additionally, they possess high fecundity where a single pair of fish can produce hundreds of offspring per week for almost no cost, allowing for large sample sizes.

The immune system of zebrafish is exceptionally well conserved compared to that of mammals. Analogous myeloid-derived immune cells including monotcytes, macrophages (of both M1 and M2 subtypes), neutrophils,

eosinophils, mast cells, and cytotoxic cells similar to NK cells have all been characterized in the zebrafish [35-39]. Hematopoiesis occurs in the developing zebrafish at 22 hpf. By 48 hpf embryos have fully competent macrophages and neutrophils that can respond to bacterial pathogens [40-42]. Embryos hatch from their chorion at roughly 3 dpf and become colonized by a microbiota at this point [43]. *Rag*-dependent rearrangements in B and T cell progenitors also occurs in this window, though functional adaptive immunity is not achieved until larvae are approximately 4 weeks old [44, 45]. Sensing the presence of microbes is paramount for being able to initiate an effective immune response, so mammals and zebrafish contain multiple families of pattern recognition receptors such as Toll-like receptors, NOD-like receptors, RIG-I-like receptors, and peptidoglycan recognition proteins [46-51]. Following the triggering of an innate immune response in zebrafish, well conserved transcriptions factors including NFkB, AP-1, IRF, and STAT are induced. There is also conservation of most cytokines and chemokines that function as important messengers of the immune system [50, 52].

The zebrafish is an attractive model for the study of a wide range of bacterial, fungal, and viral diseases [reviewed in 39, 53]. The advantages of the model are maximal during the early stages of development (days 1-7) so most of the research has focused on these stages, but adult zebrafish are also used to study host-microbe interactions. Work in zebrafish examining the pathogenesis of *Mycobacterium marinum*, a fish pathogen related to *Mycobacterium tuberculosis*,

revealed that macrophages play a dual role during infection. While they are required to curtail bacterial growth during early infection and wall off bacteria in a granuloma, they also serve to benefit the bacteria through facilitating their expansion and dissemination [54, 55]. A zebrafish embryo model of *E. coli* infection has also proved valuable in resolving diverse virulence phenotypes and niche-specific restrictions among closely related strains, including the strain's varying reliance on secreted toxins such as HlyA and CNF1 [42]. The zebrafish has also been used in combination with bacterial forward genetic screens to successfully identify novel virulence determinants in *E. coli*, *M. marinum*, and *Streptococcus pyogenes* [56-58]. Finally, a screen performed in the zebrafish to identify mutations that mediate sensitivity or resistance to *Mycobacterium* infection also proved the utility of using zebrafish to identify novel host determinants that impact infection [59].

It is only during recent years that the substantial contributions made by resident microflora have been realized. Given the relative ease in generating and maintaining gnotobiotic (germ free) zebrafish [60], it is now being used as a model to study the assembly and behavior of intestinal microbial communities and how these communities shift in response to genetic, environmental, and dietary perturbations [61-63]. Zebrafish are also an appealing model for screening chemical libraries that for the ability to modulate both microbe and host biological processes. Compounds can easily be taken up through the skin of developing embryos and drug efficacy can be assayed simultaneously with

general toxicity [64]. Additionally, when screening for antimicrobials, using an *in vivo* model system avoids complications that arise from the fact that *in vitro* microbial culture conditions are usually very different than the environment microbes encounter *in vivo* during infection.

The transparency of zebrafish embryos and larvae allows for the interactions between microbe and host to be monitored *in vivo* in real time and with enough clarity to identify morphological changes in leukocytes [65-68]. Taking advantage of transgenic lines where phagocytes are fluorescently labeled, reagents that allow for the fluorescent visualization of phagocyte respiratory burst, and fluorescently tagged *Candida albicans*, a zebrafish model was able to show for the first time *in vivo* that NADPH oxidase is required for *C. albicans* filamentation [66, 69]. Given its transparency, the zebrafish is also an attractive model for immune cell migration and chemotaxis as well as resolution of inflammation and wound healing [70-72].

Currently the use of zebrafish as a model for the study of host-microbe interactions and immunology is limited by paucity of available reagents, such as genomic disruption in immune genes, antibodies that recognize zebrafish epitopes, and other molecular tools. In Chapter 3, I will discuss the generation of several novel zebrafish lines to study the contributions of IL-1β and IL-10 to infection, inflammation, and other biological processes.

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CHAPTER 2

LETHAL STRAINS OF *ESCHERICHIA COLI* TRIGGER MARKEDLY DIVERSE HOST RESPONSES DURING SEPSIS

Abstract

Sepsis is a life-threatening systemic inflammatory condition caused by the presence of microorganisms in the bloodstream. In septic individuals, the infecting microbes are commonly viewed as generic inducers of inflammation while the host background is considered the primary variable affecting disease progression and outcome. To study the effects of bacterial strain differences on the maladaptive immune responses that are induced during sepsis, I employed a zebrafish embryo infection model using extraintestinal pathogenic *E. coli* (ExPEC) isolates. These genetically diverse pathogens are a leading cause of sepsis and are becoming increasingly problematic due to the rise of multidrug resistance strains. Zebrafish infected with ExPEC isolates exhibit many of the pathophysiological features seen in septic human patients, including dysregulated inflammatory responses (cytokine storms), tachycardia, endothelial leakage, and progressive edema. Mirroring the situation with human patients, antibiotic therapy reduced ExPEC titers and improved host survival rates, but was only effective within limited time frames, and surviving animals often developed lasting edema and other defects. However, only a limited subset of ExPEC isolates can trigger a sepsis-like state and death of the host when introduced into the bloodstream. Intriguingly, I find that phylogenetically distant, but similarly lethal, ExPEC isolates can stimulate markedly different host responses, including disparate levels of inflammatory mediators. Altogether, this work establishes zebrafish as a relevant model for key aspects of human sepsis

and highlights the ability of genetically distinct ExPEC isolates to induce divergent host responses independent of baseline host attributes.

Introduction

Escherichia coli is an incredibly diverse species, both genetically and in terms of its ability to colonize numerous niches in the environment and within animal hosts. The relationship between *E. coli* and its animal hosts can be mutualistic, as is thought to be the case for most *E. coli* strains within the intestinal tract of mammals, or pathogenic, causing diarrheal diseases, urinary tract infections, meningitis, sepsis, and other maladies. Strains that can instigate disease outside of the intestinal tract, termed extraintestinal pathogenic *E. coli* (ExPEC), are very common and have a huge impact on human health and mortality [1]. The ability of some ExPEC strains to gain access to and disseminate within the bloodstream is especially problematic and oftentimes lethal. ExPEC are the principal cause of bacteremia and a leading cause of sepsis, second only to Group B *Streptococcus* in neonates and *Staphylococcus aureus* in adults [2-5]. Over the past few decades there has been a concerning increase in the rates of *E. coli*-induced sepsis in both adults and neonates, but the pathogenesis of these infections remains for the most part undetermined.

During sepsis, the generation of excessive inflammatory mediators, including cytokines and reactive oxygen species, can result in vascular leakage, disseminated intravascular coagulation (DIC), and organ failure. Clinically, septic patients may present with highly variable symptoms that can make it difficult to

assess disease severity or predict outcomes [6, 7]. Much of the variability in disease progression in septic patients has been attributed to differences in the genetic background and immune status of individual hosts [8-10], but the nature of the infecting microbes can also impact disease outcome. For example, relative to Gram-positive pathogens and other Gram-negative bacteria, ExPEC are generally more inflammatory and more likely to cause death during sepsis [11, 12]. Animal models indicate that the survival, growth, and virulence of specific ExPEC isolates within the bloodstream can be influenced by myriad bacterial genes, including those involved in adhesion, iron utilization, metabolism, membrane transport, toxin biosynthesis, and the production and modification of lipopolysaccharide and capsules [13-19]. However, in epidemiological and genomics-based studies no single set of bacterial genes has been identified that consistently correlates with the survival or lethality of ExPEC strains within the bloodstream of the human host [10, 20-25]. These findings indicate that, as a group, ExPEC strains may utilize a diverse array of functionally redundant genes to deal with host defenses and other challenges during systemic infections.

An ExPEC isolate will typically possess about 5,000 genes, but specific gene content between individual ExPEC strains can differ by as much as 30% [26]. ExPEC genomes are mosaic assemblies of individual genes, operons, and genomic islands acquired from other microbes by horizontal gene transfer. The total number of genes that may be shared among all ExPEC isolates and related *E. coli* strains in nature is referred to as the pan-genome and is currently

estimated to total more than 16,000 [20, 26, 27]. Most of these genes are at present functionally undefined, making it often difficult to correlate bacterial virulence properties with gene function. The mosaic nature of ExPEC genomes helps explain previous observations showing that the lethality of even closely related isolates can vary markedly in animal models [23, 28, 29]. It is likewise feasible that phylogenetically dissimilar ExPEC isolates can be equally lethal, but have differential effects on host signaling pathways and inflammatory responses.

Here, I set out to define how different ExPEC isolates impact host responses during sepsis. I present a novel model for studying sepsis, showing that the inoculation of a limited number of ExPEC strains into the bloodstream of zebrafish embryos elicits many of the pathophysiological and transcriptional responses seen during human sepsis. Importantly, I find that even similarly lethal bacterial isolates can trigger notably divergent host responses, an unappreciated phenomenon that could in part account for the variability in physiological features observed in human septic patients.

Results

Few *E. coli* strains cause death following entry into the bloodstream

To investigate the potential of different ExPEC isolates to induce variable host responses during systemic infections, a panel of *E. coli* strains from various sources were injected into the bloodstream of 48-hours postfertilization (hpf) zebrafish embryos. At this developmental stage, the zebrafish immune system

consists solely of innate defenses, including macrophages, neutrophils, complement, Toll-like receptors (TLRs), antimicrobial peptides, and cytokines similar to those encoded by humans [30, 31]. Using an inoculation dose of approximately 700-1500 colony forming units (CFU) per embryo, I found that the K12 strain MG1655 and the human gut isolate HS caused no death at 24 hours postinoculation (hpi), whereas the sequenced ExPEC reference strains F11 and CFT073 were both highly lethal within the same time frame (Figure 2.1A). Two other, mostly uncharacterized ExPEC isolates (BEC7 and NMEC1) were similarly lethal. The remaining ExPEC strains tested were for the most part nonfatal, with the exception of the pyelonephritis isolate 536, which killed about 40% of the fish. In total, of all the *E. coli* strains tested, only about 26% killed more than 30% of the zebrafish following injection into the bloodstream.

In zebrafish, the lethality of an *E. coli* strain generally correlates with its ability to survive and multiply within the host [28]. This trend holds true here. I found that titers of the nonlethal strains MG1655 and HS were greatly reduced within 12 hpi, while numbers of the nonlethal ExPEC isolate UTI89 remained relatively stable during the same time frame (Figure 2.1B). In contrast, the lethal reference ExPEC strains F11 and CFT073 not only persisted within the zebrafish host, but also multiplied on average more than 1,000-fold by 12 hpi. Of note, all of the *E. coli* strains used here grew at similar rates in broth culture at 28.5°C, the temperature at which the zebrafish embryos are maintained (Figure 2.2A), and 37°C (Figure 2.2B). Taken together, these results highlight the strain-dependent

lethality of *E. coli* following inoculation into the zebrafish bloodstream, and are in line with previous observations indicating that the bloodstream is a highly restrictive environment for many bacteria [13, 28, 32].

Differential pathophysiological effects of similarly lethal but phylogenetically distant ExPEC isolates

F11 and CFT073 are phylogenetically distinct strains as determined by multilocus sequence typing (MLST) and, although they both encode the same K2 capsular and O6 surface antigens, they possess distinct flagellar antigens, different genomic islands, and only partially overlapping sets of recognized virulence factors [27, 28, 33]. Gene content between F11 and CFT073 are about 73% identical at the protein coding level. Though these two strains are similarly lethal and grow at comparable rates within the zebrafish host, embryos infected with F11 appeared qualitatively more debilitated than embryos infected with CFT073. Infection with both strains led to overt signs of illness, including pericardial edema, ulceration of the skin, and erosion of the tail fin when compared to PBS-injected control fish (Figure 2.3A-B). However, these phenotypes were markedly more pronounced and common in fish infected with F11.

Infection with F11 also resulted in the development of prominent tissue protrusions on the trunk or tail of the embryo (Figure 2.3C). These protrusions were found on up to 50% of fish infected with F11, but were not seen with CFT073-infected embryos. Infection with F11 carrying a plasmid encoding

destabilized green fluorescent protein (F11/pGEN-GFP(LVA)) revealed that the protrusions themselves did not contain bacteria, but within the tissues and vessels adjacent to the protrusions small bacterial clusters were often detected (Figure 2.3D). The protrusions stained strongly with the fluorescent cationic dye acridine orange, which accumulates within the nucleus and cytoplasm of apoptotic cells, but not necrotic or viable cells (Figure 2.3E) [34]. Additional experiments using the fluorescently labeled transgenic zebrafish lines Tg(*krt8*:GFP) [35] and Tg(*fli1a*:EGFP) [36] showed that the F11-induced protrusions were comprised largely of epithelial cells (Figure 2.3F) and devoid of leukocytes and endothelial cells (Figure 2.3G). Staining with phalloiden to detect F-actin demonstrated that the actin cytoskeleton is significantly perturbed in these lesions and contain activated caspase-3 positive cells (Figure 2.4). These epithelial protrusions presumably arise in response to an as-yet undefined toxin or other factor(s) that is expressed by F11, but missing in CFT073.

Ciprofloxacin administration is only effective for a short window of time that varies dependent on the infecting strain

The survival of septic patients is often dependent upon rapid diagnosis and the timely delivery of appropriate antibiotics—for every hour that antibiotic treatment is delayed there is a 6-7% increased chance of patient death [37]. Even with adequate antimicrobial therapies that curtail bacterial growth, complications such as edema, immunosuppression, and coagulation defects are

frequent [38-40]. To determine how septic zebrafish respond to antibiotic treatment, I administered ciprofloxacin at various time points postinoculation with CFT073 or F11. Ciprofloxacin was chosen because it is of use clinically for the treatment of Gram-negative sepsis, particularly for sepsis arising from the gastrointestinal or urinary tract [41, 42]. Additionally, ciprofloxacin is very cell permeable, making it easy to administer by placing infected zebrafish embryos in water containing the antibiotic. When F11-infected embryos were treated with 50 μg/ml ciprofloxacin at 6 hpi, the antibiotic was able to rescue all embryos from lethal infection with no apparent complications (Figure 2.5A). When given at 12 hpi, ciprofloxacin was only slightly less effective, but a delay of just another 3 hours resulted in greatly increased mortality rates.

In sharp contrast to results obtained with the F11-infected embryos, the complete rescue of all CFT073-infected zebrafish required that I administer ciprofloxacin at a much earlier time point (3 hpi, Figure 2.5B). The addition of ciprofloxacin to CFT073-infected fish at 9 hpi decreased the survival rate to less than 30%, while waiting just another 3 hours was almost entirely ineffective (Figure 2.5B). Of note, CFT073 and F11 are similarly susceptible to ciprofloxacin as measured by both disc diffusion antibiotic sensitivity assay at 28.5°C and 37°C (Figure 2.5C and D). Furthermore, within zebrafish embryos the growth of both pathogens is effectively inhibited by addition of the antibiotic (Figure 2.5E-F). Taken together, these results demonstrate that, as with human sepsis patients, the early administration of antibiotic therapy is crucial for host survival

and that even small delays in treatment can drastically increase mortality rates. In addition, these data show that ExPEC isolates that are similarly sensitive to an antibiotic can have drastically different effects on disease outcome when exposed to the same antibiotic within the host environment.

Infected embryos often develop profound edema
following rescue with ciprofloxacin

In our studies with ciprofloxacin given to F11-infected zebrafish at 12 hpi, I noticed that the surviving animals often displayed marked pericardial edema at 24 hpi (Figure 2.6A). By 48 hpi, approximately 50% of the surviving embryos developed profound widespread edema (Figure 2.6B and C). These embryos were not retained beyond 96 hpi, but it is unlikely that they would have successfully survived to adulthood. I also observed similarly severe edema in many of the surviving CFT073-infected fish that were dosed with ciprofloxacin at 6 or 9 hpi. These results indicate that complications like subcutaneous and body-cavity edema, which are not uncommon in human sepsis patients [38, 39, 43], can also develop in zebrafish despite effective inhibition of pathogen growth.

Lethal ExPEC isolates can trigger divergent
host transcriptional responses

The differential pathophysiological effects that F11 and CFT073 have on the zebrafish host were examined more closely by transcriptional profiling. Using a previously described Agilent microarray that is enriched with probes specific for

zebrafish homologs of mammalian immunity genes [44-46], the host response to each ExPEC strain was surveyed at 6 and 12 hpi. The 6-hour time point represents the more acute phase of infection, while the 12-hour time point reflects a later stage where the two strains have established a solid foothold and replicated to high titers within the zebrafish host (see Figure 2.1B). For these experiments, groups of 18-20 embryos were injected with CFT073, F11, or a similar volume of sterile PBS as a control. RNA was then isolated from each pool of embryos at 6 and 12 hpi and processed for analysis using two-color 44k microarrays. Each experiment was repeated in biological quadruplicate. Gating on probes that were differentially expressed at least 2-fold up or down when compared to PBS-injected fish and with a *p*-value of ≤ 0.05, I found that infection with F11 altered mRNA levels for nearly a third more genes than CFT073 at both 6 and 12 hpi (Fig 2.7A and B, see also Tables 2.4-2.7). While infection with both strains changed the expression of a large number of overlapping host genes, F11 infection also induced an almost equal sized cohort of additional host genes that was not seen in CFT073-infected fish.

The biological attributes of the host genes that are differentially expressed in response to F11 and CFT073 at 6 and 12 hpi were assessed by gene ontology (GO) and KEGG pathway enrichment analysis using DAVID bioinformatics resources [47]. GO categories that were enriched at both time points with both ExPEC isolates included those dealing with host response to oxidative stress (e.g., peroxidases), immune and defense responses (e.g., cytokine expression

and complement components), proteolysis (e.g., caspases and matrix metalloproteinases), and aminoglycan metabolism (e.g., peptidoglycan recognition protein 5) (Figure 2.7C). In contrast, the numbers of genes within the GO category for regulators of transcription were significantly elevated only at 6 hpi, whereas GO categories linked with oxidation reduction (redox) reactions and blood coagulation were not enriched until 12 hpi. The latter group includes upregulated genes that encode coagulation factor IIIb (*f3b*) and the α, β, and γ chains of fibrinogen. These gene products are functionally well conserved among vertebrates as mediators of thrombosis and have been implicated in DIC during sepsis [40, 48-50].

In many cases, the numbers of differentially expressed genes within each enriched GO category were greater for the F11-infected fish (Figure 2.7C). This trend was also observed by KEGG pathway analysis (Figure 2.8). Common pathways affected by F11 and CFT073 at both 6 and 12 hpi include those involved in apoptosis, proteasome activities, arachidonic acid metabolism, and TLR, MAP kinase, and cytokine signaling. Pathways associated with primary bile acid metabolism were also significantly altered by both pathogens, though CFT073 was slower to induce the changes. Other host metabolic pathways, including those connected with the metabolism of select sugars and amino acids, were differentially affected by F11 and CFT073 only at 12 hpi. At this time point F11 also caused significant transcriptional changes in several genes that encode proteins within the p53 pathway, which has a central role as a regulator of host

cell cycle arrest and apoptosis in response to stress [51]. Altered levels of factors like Cyclin B1, GADD45 homologues, and Sestrin-2 within the p53 pathway may enable host cells to better sense and deal with oxygen radicals and other genotoxic stresses generated during the infection process [52-55], but could also conceivably help promote the formation of the apoptotic epithelial protrusions seen in many F11-infected embryos.

Zebrafish embryos infected with lethal ExPEC isolates have transcriptional responses and pathologies like those seen in human sepsis

In humans with sepsis or related syndromes like endotoxemia, there is often a large amount of variation in the gene expression datasets obtained from different studies [56]. However, general trends in these datasets are discernable and are reiterated, in large part, in our zebrafish infection model (Table 2.1). These include the upregulation of Pattern Recognition Receptors like TLR5 as well as components of the NFκB, MAP kinase, and Jak-STAT signaling pathways. Activation of these pathways can stimulate the expression of multiple cytokines, chemokines, and other immunomodulatory factors. The high level expression of numerous cytokines and other inflammatory mediators induced in zebrafish by both F11 and CFT073 is reminiscent of the cytokine storms seen in human patients during sepsis. Major inflammatory cytokines that are substantially elevated both in our assays and in human patients with sepsis-like syndromes include TNFα, TNFβ, IL-1β, IL-6, IL-8, IL-12, and IL-17 [48, 57-62]. During human

sepsis, as in many inflammatory diseases, there is imbalance among pro- and anti-inflammatory mediators [62]. We observe a similar situation in our zebrafish model, as evidenced by the simultaneous upregulation of multiple pro-inflammatory signals along with several key anti-inflammatory factors, including IL-10, SOCS1 and 3, IRAK3, and galectin-1 (Table 2.1).

Clinically, sepsis is diagnosed when there is a documented source of infection as well as at least two of the following signs: fever (temperature above 38°C) or hypothermia (temperature below 35.5°C), tachypnea (more than 20 breaths per minute), hypocapnia ($PaCO_2$ of less than 32 torr), tachycardia (more than 90 beats per minute), and leukocytosis (more than 12,000/mm^3) or leukopenia (less than 4,000/mm^3) [37]. Additional pathologies commonly seen during sepsis include endothelial dysfunction and consequent loss of barrier function leading to progressive subcutaneous edema and clotting abnormalities, including DIC. Considering these criteria, I set out to determine if zebrafish embryos infected with ExPEC display any overt signs of sepsis like those seen clinically in human patients.

Given that changes in body temperature are less applicable in ectothermic organisms such as zebrafish, I did not evaluate fever or hypothermia in ExPEC-infected embryos. ExPEC-induced leukopenia in zebrafish is suggested by previous work from our group in which we note that F11- or CFT073-infected zebrafish have notably diminished numbers of phagocytes by 12 hpi, relative to MG1655-infected controls [28]. As a measurement of vascular leakage, I injected

fluorescent dextran (70 kDa) into the bloodstream of embryos and quantified its movement from the vasculature into host tissues (myotomes) after 15 min (Figure 2.9A). In comparison with control embryos that were injected with nonpathogenic MG1655 or PBS, embryos that were infected with F11 had significantly higher amounts of vascular leakage by 9 hpi (Figure 2.9B). Disruption of endothelial barriers may be driven in part by heightened cytokine levels and the abundant expression of MMP9 and other metalloproteinases in response to ExPEC. These events may also contribute to the edema that becomes manifest within the pericardial cavity and at other sites within ExPEC-infected embryos at later time points (see Figures 2.2A, 2.6A, and 2.6C). Heart rate measurements showed that ExPEC can also induce tachycardia during systemic infections at 6 hpi (Figure 2.9C), though heart rates eventually decline as the embryos succumb to the infection. Interestingly, our microarray analysis indicates that at 12 hpi both CFT073 and F11 induce expression of UCP2, a mitochondrial uncoupling protein that is linked with stress hyperglycemia and heart failure in human sepsis patients [63, 64]. In total, these observations demonstrate that ExPEC-infected zebrafish have altered gene expression patterns and develop overt pathologies on par with those that are often observed in humans with sepsis.

F11 and CFT073 have differential effects on host TLR signaling and associated inflammatory responses

Our KEGG analysis indicated that F11 and CFT073 have especially sizeable effects on TLR signaling pathways. A map that illustrates how F11 and

CFT073 influence the transcription of specific TLR genes, associated signaling factors, and various downstream targets is presented in Figure 2.10. Both pathogens alter the expression of several key components within canonical MyD88-dependent and MyD88-independent TLR signaling cascades, as well as intersecting and overlapping MAP kinase, Jak-STAT, Interferon (IFN), and Tumor Necrosis Factor (TNF) signaling pathways. Although F11 and CFT073 have similar effects on expression of many of the inflammatory mediators depicted in Figure 2.10, our array data indicate that the pathogens can also elicit conspicuously divergent inflammatory responses. Examples of this phenomenon are seen with two conserved cytokines—the macrophage-activating cytokine IFN-γ2 (*ifng1-2*), which is induced by F11 but repressed by CFT073, and the anti-inflammatory factor IL-10, which is markedly upregulated by CFT073 but less so by F11.

qRT-PCR was used to validate a subset of our microarray results and to explore the host transcriptional responses to additional *E. coli* strains (Figures 2.11 and 2.12). In agreement with our microarray data, qRT-PCR indicated that CFT073 induced significantly higher levels of IL-10 than did F11 at both 6 and 12 hpi (Figure 2.11A). Following a spike at 6 hpi, IL-10 transcript levels in CFT073-infected fish were reduced by 12 hpi to levels seen in embryos injected with either the gut isolate HS or the nonlethal ExPEC strain UTI89. Interestingly, the IL-10 mRNA levels induced by HS and UTI89 at 12 hpi were still significantly greater than those observed in F11-infected fish and in control animals that were

inoculated with either PBS or the K12 strain MG1655. The situation was quite different when transcript levels for the pro-inflammatory cytokines Il-1β and IL-8 were quantified. Relative to the nonlethal strains, CFT073 and F11 enhanced the expression of IL-1β and IL-8 similarly at 6 hpi, but by 12 hpi the transcript levels for these cytokines in F11-infected embryos were markedly higher than those in fish infected with CFT073 (Figure 2.11B-C). Similar trends with somewhat less pronounced differences between F11- and CFT073-infected fish were observed with the pro-inflammatory cytokines TNFα, Interferon-1 (IFN-1), and IL-6 (Figure 2.11D-E, and Figure 2.13E). In contrast, F11 and CFT073 had comparable effects on the expression of another pro-inflammatory cytokine, IL-17c, which was strikingly elevated at both 6 and 12 hpi only in response to the two lethal pathogens (Figure 2.11F).

The differing effects of F11 and CFT073 on cytokines like IL-1β and IL-8 were in part recapitulated when I used qRT-PCR to survey expression levels of three other host defense genes—*saa1*, *hamp1*, and *mmp9*. Transcription of the gene encoding serum amyloid A1 (SAA1), which is an acute phase protein with antimicrobial activity [65-68], was significantly induced by all tested *E. coli* strains, relative to PBS-injected controls (Figure 2.12A). However, F11 caused a more rapid and higher level of induction than any of the other strains, including CFT073. Transcript levels for hepcidin (HAMP1), which is an antibacterial peptide that also functions as a key regulator of iron homeostasis [69], were significantly elevated at 6 hpi in response to all strains except MG1655 (Figure 2.12B). By 12

hpi, *hamp1* expression was notably higher in the F11-infected embryos than in all other samples, including the CFT073-infected fish. A comparable expression pattern was seen with the matrix metalloproteinase MMP9, a secreted protease that facilitates the migration of leukocytes to sites of infection (Figure 2.12C) [70]. Of note, serum levels of SAA1, hepcidin, and MMP9 are elevated during human sepsis and have been investigated as prognostic biomarkers [71-73].

Overall, these qRT-PCR results are in close agreement with our microarray data, demonstrating that similarly lethal ExPEC isolates like F11 and CFT073 can elicit distinctive, though overlapping, inflammatory responses during systemic infections. This phenomenon was mirrored in a mouse model of sepsis in which I focused on IL-6 expression. IL-6 is a marker of sepsis severity, with elevated levels of this cytokine during early sepsis correlating with increased mortality rates in both humans and mice [74-76]. Following subcutaneous inoculation of adult outbred Swiss Webster mice with 10^8 CFU of either CFT073 or F11, both pathogens disseminate systemically and kill 90-100% of the host animals within 24 hours. At 6 and 12 hpi, I detected no significant differences in the titers of the two ExPEC isolates within the kidneys, liver, or spleen (Figure 2.13A-C). Relative to PBS-injected controls, both pathogens induce production of IL-6, but by 12 hpi the levels of IL-6 in the sera of F11-infected mice were significantly higher than those in CFT073-infected animals (Figure 2.13D). This pattern of IL-6 expression was also seen, though with less statistical significance, in F11- and CFT073-infected zebrafish embryos (Figure 2.13E), paralleling our

findings with other pro-inflammatory cytokines.

Discussion

Septic patients often present with wide-ranging disease symptoms, and are notoriously difficult to manage because they do not respond homogenously to treatment [56]. In septic individuals, the infecting microbes are commonly viewed as generic inducers of inflammation while the host background is considered the primary variable affecting disease progression and outcome. Here, using a model system in which bacteria are injected into the bloodstream of zebrafish embryos, I found that only a limited subset of ExPEC isolates is able to cause overt sepsis-like disease. Most strikingly, this work demonstrates that similarly lethal bacterial isolates belonging to the same species can affect the development of sepsis in markedly different ways irrespective of host background characteristics.

Our results support previous observations indicating that the bloodstream is a restrictive niche for most *E. coli* strains [13, 28]. The survival and growth of ExPEC during systemic infections are likely limited by nutrient and iron availability and the presence of various antimicrobial factors such as circulating phagocytes, complement, and antimicrobial peptides. Recent functional genetic screens from our group and others show that numerous bacterial genes can promote the fitness of ExPEC strains within the bloodstream [13-15, 32]. However, many of the genetic loci implicated in ExPEC survival during systemic infections remain functionally indeterminate. Rather than always relying on specific sets of homologous genes, it appears that ExPEC strains, as a group,

may instead employ multiple nonorthologous, but functionally redundant, factors to deal with the myriad challenges encountered within the bloodstream. The capacity of ExPEC isolates to evolve and utilize distinct solutions to challenges presented by the host likely contributes to the ability of ExPEC strains to differentially affect host responses during sepsis.

In our assays, F11 altered the transcription of nearly a third more host genes than CFT073 at 6 and 12 hpi, while simultaneously inducing higher-level expression of many pro-inflammatory cytokines and other immunomodulatory factors. This trend was also observed with serum IL-6 levels in a mouse sepsis model. In comparison with CFT073-infected zebrafish, those infected with F11 often appeared qualitatively more ill and frequently developed apoptotic epidermal protrusions. These may be manifestations of the cutaneous lesions seen in some human patients with bacteremia or sepsis [77], but the factors that promote their formation are not yet defined. Overall, CFT073 was less inflammatory than F11 and is arguably a stealthier pathogen, stimulating the expression of more anti-inflammatory factors like IL-10 and producing fewer overt signs of illness during the initial stages of infection. From these results, I posit that the ability of different ExPEC isolates to elicit dissimilar host inflammatory responses in human patients contributes to the heterogeneity of symptoms experienced by septic individuals.

Though F11 and CFT073 encode many of the same fitness and virulence determinants, including the same capsular K2 and LPS O6 surface antigens, they

each have a distinct repertoire of known virulence genes. For example, each pathogen expresses the repeat-in-toxin (RTX) family member α-hemolysin (HlyA), but only CFT073 encodes the RTX protein TosA (a.k.a. UpxA). In zebrafish, HlyA acts to disable phagocytes while TosA functions as an adhesin that can promote bacterial survival during systemic infections [16, 28]. Both F11 and CFT073 also carry homologues of the serine protease autotransporter toxins Tsh and Vat, but only CFT073 expresses a related toxin known as Sat. Of note, in a recent large-scale sequencing study of 312 ExPEC strains, *sat* and *tosA* genes were enriched among isolates from bacteremic patients [20]. Yet results presented here with F11 show that these genes are not strictly required for ExPEC survival and growth within the bloodstream. The fitness of F11 during systemic infections is, however, increased by another secreted protein known as cytotoxic necrotizing factor (CNF1). This toxin, which is absent in CFT073, causes the constitutive activation and eventual degradation of host Rho GTPases [78]. The deletion of *cnf1* significantly diminishes the growth and virulence of F11 within the zebrafish host (Figure 2.14). Altered expression of IL-1β (Figure 2.15A) and MMP9 (Figure 2.15C) were seen in the *cnf1* mutant compared to F11 at 12 hpi, but these data are somewhat difficult to interpret given the significant difference in bacterial titers at this time point (Figure 2.14B). Furthermore, the mutant bacteria can still trigger the formation of epidermal protrusions.

A number of ExPEC-associated proteins that can be described as immunomodulators may also account for the differential effects of F11 and

CFT073 on the transcription of host pro- and anti-inflammatory mediators. Both CFT073 and F11 have genes for the TIR domain-containing protein TcpC, which can antagonize host TLR signaling [79]. A major TLR affected in our zebrafish model, as well as in some human patients with sepsis-like syndromes, is the flagellin receptor TLR5 [59, 61]. CFT073 and F11 produce distinct flagellar antigens (H1 and H31, respectively), which could potentially impact how these two pathogens engage TLR5 and influence downstream signaling cascades. CFT073, but not F11, may further affect host inflammatory pathways via expression of the inflammation repressors SisA/B and the neutrophil migration suppressor YbcL [80, 81].

The diversity of host responses elicited by F11, CFT073, and other ExPEC isolates in our zebrafish model reflects many of those seen in human patients with bacteremia or sepsis. Overt pathophysiological changes observed within the infected zebrafish and in human septic patients include tachycardia, vascular leakage, edema, and signs of leukopenia. In addition, though we did not assess clotting abnormalities directly, our microarray results show that the ExPEC strains do have significant effects on host coagulation factors at 12 hpi, suggesting that the pathogens may cause disregulation of clotting cascades that could lead to problems like DIC.

Since 2000, hospitalization rates for sepsis have doubled and associated costs have skyrocketed in the USA to greater than $20 billion annually [82]. In the clinic, the effective treatment of sepsis typically requires early diagnosis and the

timely delivery of fluids to maintain blood pressure plus broad-spectrum antibiotics to control the infecting bacteria [37, 83]. Results presented here reiterate the importance of timely antibiotic delivery for survival of the septic host (see Figure 2.9). Furthermore, our data show that the window during which antibiotic therapy can effectively rescue the host can vary greatly, even when comparing ExPEC strains that are equally sensitive to the drug *in vitro*. Adding to the complexity of factors that can confound the assessment and treatment of septic patients is the ongoing rise of Multidrug Resistant (MDR) pathogens, including MDR ExPEC strains [84, 85].

Zebrafish embryos are amenable to high-throughput pharmacological and genetic screens, and work from our group has already demonstrated the utility of zebrafish as a tool for identifying and functionally defining ExPEC-associated factors that are of importance in mammalian hosts [13, 14, 16, 28, 86]. Considering the tractability of the zebrafish infection model and its ability to recapitulate important aspects of human disease, as shown here, I expect that it will provide a useful platform for the examination of cellular processes during sepsis and the much needed discovery of improved therapies for sepsis and associated sequelae.

Materials and Methods

Ethics statement

Animals used in this study were handled in accordance with University of Utah and IACUC-approved protocols following standard guidelines described at

www.zfin.org and in the Guide for the Care and Use of Laboratory Animals, 8th Edition.

Bacterial strains

The bacterial strains used in this study are listed in Table 2.2. Bacteria used for infecting zebrafish and mice were grown statically at 37°C for 24 h in 12 ml modified M9 minimal medium (6 g/l Na_2HPO_4, 3 g/l KH_2PO_4, 1 g/l NH_4Cl, 0.5 g/l NaCL, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 0.1% glucose, 0.0025% nicotinic acid, 0.2% casein amino acids, and 16.5 µl thiamine in H_2O, pH 7.2). F11 carrying pGEN-GFP(LVA) was grown in medium containing ampicillin (50 µg/ml). Targeted deletion of *cnf1* in F11 was generated using lambda Red-based recombination and the primers noted in Table 2.3, as described previously [87].

Zebrafish embryos

Wild type AB zebrafish and the transgenic lines Tg(*krt8*:GFP) and Tg(*fli1a*:EGFP) were maintained as breeding colonies on a 14-h/10-h light/dark cycle. The Tg(*krt8*:GFP) fish express GFP in the outermost layer of epithelial cells while the Tg(*flia1*:EGFP) fish have EGFP-labeled endothelial cells and leukocytes [35, 36]. Embryos were collected as mixed egg clutches and raised at 28.5°C in E3 medium (5 mM NaCl, 0.27 mM KCl, 0.4 mM $CaCl_2$, 0.16 mM $MgSO_4$; pH 7.4) containing 0.000016% methylene blue as an antifungal agent.

Infection of zebrafish embryos

48 hpf embryos were manually dechorianated, briefly anesthetized with 0.77 mM ethyl 3-aminobenzoate methanesulfonate salt (tricaine) (Sigma-Aldrich), and embedded in 0.8% low melt agarose (MO BIO Laboratories) without tricaine. After the agarose solidified, embryos were immersed in E3 media lacking methylene blue. Prior to injection, 1 ml of bacterial culture was pelleted, washed with 1 ml sterile PBS, and resuspended in PBS to obtain ~1X10^9 CFU/ml PBS. One nl of this bacterial suspension was microinjected into the bloodstream via the circulation valley using an Olympus SZ61 or SZX10 stereomicroscope together with a YOU-1 micromanipulator (Narishige), a Narishige IM-200 microinjector, and a JUN-AIR model 3-compressor. For each experiment, the average CFU per injection was determined by adding 10 1-nl drops to 1 ml of 0.7% NaCl, which was then serial diluted and plated on Luria-Bertani (LB) agar plates. Mock-infected controls were inoculated with 1 nl sterile PBS. Following injection, embryos were removed from agar and placed individually into wells of a 48-well plate (Nunc) containing E3 medium and incubated at 28.5°C. For lethality assays, death was defined by absence of heart contraction and blood flow.

Enumeration of bacterial numbers in zebrafish embryos

Embryos were homogenized at the indicated time points in 500 µl of PBS containing 0.5% Triton X-100 using a mechanical PRO 250 homogenizer (PRO Scientific). Homogenates were serially diluted and plated on LB agar plates, which were then incubated overnight at 37°C.

Growth curves

Bacteria were grown from frozen stocks at 37°C with shaking overnight in 5 ml of LB broth or modified M9 minimal medium (6 g/liter Na_2HPO_4, 3 g/liter KH_2PO_4, 1 g/liter NH_4Cl, 0.5 g/liter NaCl, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 0.1% glucose, 0.0025% nicotinic acid, 16.5 µg/ml thiamine, and 0.2% casein amino acids). Cultures were then diluted 1:100 into the indicated medium, and the growth of quadruplicate 200-µl samples in shaking 100-well honeycomb plates at 37°C was assessed using a Bioscreen C instrument (Growth Curves USA).

Mouse sepsis model

Outbred female Swiss Webster mice (Charles River) that were 5-6 weeks old were briefly anesthetized using isofluorane inhalation and injected subcutaneously with 10^8 CFU of bacteria in 200 µl warm, sterile PBS. After 6 or 12 h, mice were euthanized and the kidneys, livers, and spleens were collected and weighed. Excised tissues were homogenized in PBS containing 0.5% Triton X-100 using a Bullet Blender Storm 24 (Next Advance) in tubes containing 3.2 mm stainless steel beads. These beads disrupt host tissues, but do not affect bacterial viability. Homogenates were serially diluted and plated on LB agar plates for determination of bacterial titers.

Quantification of mouse serum IL-6 levels

Blood was obtained from mice by cardiac puncture at the time of euthanasia. IL-6 concentrations in sera collected from the blood samples were

determined by sandwich ELISA using clone MP5-20F3 as a capture antibody and clone MP5-2C11 as a detection antibody. Recombinant IL-6 was used as a standard. Antibodies for these assays were purchased from BD Pharmingen.

RNA isolation from zebrafish embryos

Pools of 15-20 embryos were manually homogenized in 1 ml QIAzol Lysis Reagent (Qiagen) and total RNA extracted using the Qiagen RNeasy Plus Universal Kit according to the manufacturer's instructions. Genomic DNA was removed by the gDNA Eliminator Solution (Qiagen). Samples used for microarray and qRT-PCR had a minimum RNA integrity number score of 9.

Microarray design, labeling, and hybridization

Microarray analysis was performed with biological quadruplicate samples using custom-designed 44k Agilent chips [44-46]. Fluorescently labeled cRNA was synthesized using the Two-Color Low RNA Input Linear Amplification Kit (Agilent). Fluorescently labeled cRNA (825 ng) was then fragmented, combined with Hi-RPM Hybridization Buffer (Agilent), and hybridized using a SureHyb Hybridization chamber (Agilent). Slides were scanned in a G2505C Microarray Scanner (Agilent). Labeling, hybridizing, and scanning were performed by the Microarray and Genomic Analysis Core at the University of Utah.

Microarray data analysis

TIFF files of the scanned microarrays were processed using Feature Extraction Software version 10.5 (Agilent). Array data was then log2 transformed,

Lowess-normalized, and median-centered. Fold changes were calculated by comparing infected samples to mock-infected (PBS-injected) controls from the same clutch of embryos. Significance cutoffs were defined as an average of ≥ 2.0 fold change across all 4 arrays and $p \leq 0.05$. In order to restrict the false discovery rate, all *p*-values were adjusted using the Benjamini and Hochberg method [88]. GO and KEGG analyses were performed using Database for Annotation, Visualization, and Integrated Discovery (DAVID) (http://david.abcc.ncifcrf.gov/) as described [47].

cDNA synthesis and qRT-PCR

cDNA was synthesized from 2 µg of RNA using the SuperScript III First-Strand Synthesis System (Invitrogen) in a 20 µl reaction volume. Following assembly of the master mix according to manufacturer instructions, samples were incubated at 25°C for 5 min, 55°C for 45 min, and 70°C for 15 min. Complimentary RNA was then removed by addition of RNase H (Invitrogen) for 15 min at 37°C.

qRT-PCR was performed on a LightCyler 480 instrument (Roche) following manufacturer recommendations. Cycling parameters were 95°C for 8 min to activate the polymerase followed by 40 cycles of 95°C for 4 sec, 60°C for 6 sec, and 72°C for 6 sec. Fluorescence measurements were taken at the end of each cycle. Melt curve analysis was performed to ensure that no primer dimers were amplified. All reactions were performed in technical duplicate. Sequences for the forward and reverse primers are listed in Table 2.3. Results were

normalized to transcript levels of the housekeeping gene *ef1*α (elongation factor 1-alpha) using $\Delta\Delta C_t$ analysis.

Zebrafish staining

To assess host cell viability, 12 hpi wild type zebrafish embryos were incubated for 30 min at 28.5°C in E3 medium with 10 μg/ml acridine orange. Samples were then washed several times with E3 medium and embedded in low melt agar for imaging. To further investigate the cell biology of the tissue protrusions, embryos were fixed in 4% paraformaldehyde with 0.2% Triton X-100, blocked in PBS containing 10% heat inactivated goat serum and 0.5% Triton X-100, and stained with active caspase-3 (Abcam) and Alexa Fluor 594-phalloidin (Abcam) overnight with three washes in 0.5% Triton X-100 in PBS between each step. Tails were then cut and mounted between a coverslip and slide in ProLong Gold (Invitrogen).

Zebrafish imaging

Embryos were imaged using a fluorescent Olympus SZX10 stereomicroscope equipped with an Olympus DP72 camera or on a Nikon Eclipse TE300 inverted microscope fitted with spinning disc confocal system (Andor Technologies) and images captured using a Micromax charge-coupled device camera (Roper Scientific).

Heart rate measurements in zebrafish embryos

Infected and PBS-injected control zebrafish embryos were briefly anesthetized with 0.77 tricaine (Sigma-Aldrich), embedded in low melt agarose (MO BIO Laboratories) without tricaine, and immersed in E3 media lacking methylene blue. After 1 h, embryos were filmed for ~1 min using an Olympus SZX10 stereomicroscope and an Olympus DP72 camera recording at 15 frames/sec. Heart rates (beats/min) were calculated from review of the footage.

Quantifying endothelial leakage in zebrafish embryos

Embryos were injected intravenously with 1 nl of tetramethylrhodamine-conjugated, lysine-fixable 70 kDa dextran (25 μg/ml; Life Technologies). After 15 min, embryos were fixed overnight at 4°C in PBS containing 4% paraformaldehyde. Samples were then washed 3X with PBS and taken through a series of washes with 30%, 50%, and 80% glycerol before mounting on slides using FluorSave Reagent (Calbiochem). Embryos were imaged using a fluorescent Olympus SZX10 stereomicroscope and an Olympus DP72 camera, ensuring equal exposure times for each sample. Using ImageJ [89], the mean fluorescent intensity for 3 specific myotomes, as well the lumen of the caudal artery, was measured (see Figure 9A). Endothelial leakage was defined as the ratio of the mean fluorescent intensity in a myotome to the mean fluorescent intensity in the lumen of the caudal artery below that myotome. This method accounts for variability in the amount of dextran injected.

Zebrafish ciprofloxacin assays

At the indicated time points, infected zebrafish embryos were transferred from E3 medium into E3 medium containing 50 μg/ml ciprofloxacin (pH 7.4). For enumeration of bacterial titers within ciprofloxacin-treated embryos, the fish were washed with E3 medium twice to remove ciprofloxacin and then homogenized and plated as described above.

Statistical analysis

Except where indicated, *p* values were calculated by two-tailed Student's t-tests, Mann-Whitney U tests, or log-rank tests using Prism 6.0e software (GraphPad Software). Values of less than 0.05 were defined as significant for all experiments.

Acknowledgments

I thank ARUP Laboratories, Hillary Crandall (Primary Children's Hospital, Salt Lake City, UT), and the Andrew Weyrich lab (University of Utah, Salt Lake City, UT) for providing bacterial strains. I am also grateful to Herman Spaink (University of Leiden, Netherlands) for kind use of their microarray design. I would also like to thank Brittany Fleming for her assistance on the growth curve and serum resistance assays.

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Table 2.1. Gene sets that are differentially expressed in zebrafish embryos due to systemic infection with ExPEC are functionally similar to many of those that are changed in human patients with sepsis.

Functional Category	Gene Products*	Links to Human Sepsis**
Signal transduction		
Pattern Recognition Receptors	Tlr5, MARCO scavenger receptor, MyD88, IRAK4, IRF7, TRAF1/2b/3	[58-61, 90]
NFκB	Rel, NFκB2, NFκBIa (IκB-alpha), Bcl3, TRAF1/2b, RIPK2	
MAP Kinase	Fos, ATF-3, Jun, MEKK5, TRAF1/2b, GADD45A/B	
Jak-STAT	Jak1, STAT4, STAT1b, STAT3, IRF9	
Pro-inflammatory mediators	TNFα/β, TNF Receptors (TNFRSF1a and TNFRSF9a), IL-1β, IL-12, IL-17, IL-8	[48, 58, 59, 61]
Anti-inflammatory factors	SOCS1/3, IL-10, Galectin-1, IRAK3 (IRAK-M)	[58-61, 90, 91]
Acute Phase Proteins	SAA1, Hepcidin (HAMP1), Haptoglobin, Hemopexin	[48, 59, 60, 71, 73, 90, 92]
Coagulation and Complement	Coagulation factor IIIb, Fibrinogen, Complement factor B, Clusterin, Complement components C3b, C3c, C4-2, C6, C7-1	[59],[60, 90]
Protease activities	MMP9/13/30, Proteasome subunits, Cathepsin C/H, Carboxypeptidase A4, ADAM8a, SERPINB1/5	[59, 60, 72]
ROS generation and management	Neutrophil Cytosolic Factor 1 (p47-PHOX), Cytochrome b-245 (p22-PHOX), Glutathione peroxidase 1b, Thioredoxin, UCP2, GADD45A/B, Metallothionein 2, Solute Carrier Family 30/39	[49, 58, 59, 61, 63]
Apoptosis	Caspase 8, Fas, MEKK5 (ASK-1), CFLAR, TRAF1/2b, Galectin-1, RIPK2	[58-61]
Arachidonic acid metabolism	Prostaglandin-endoperoxide synthase 1 (COX-1), Prostaglandin-endoperoxide synthase 2a/b (COX-2), Glutathione peroxidase 1b, Epoxide hydrolase 2, Cytochrome p450s, Cholesterol 25-hydroxylase	[59, 93, 94]

* Transcript levels for the indicated gene products are significantly increased ≥ 2-fold (p<0.05) in F11- and/or CFT073-infected zebrafish embryos at 6 and/or 12 hpi, relative to mock-infected controls.

** References are for studies that implicate the specified functional categories or select gene products in human sepsis and related syndromes, but do not necessarily represent all pertinent publications

Table 2.2. Bacterial strains and plasmids used in Chapter 2.

***E. coli* Strain**	**Description**	**Source or Reference**
F11	Cystitis isolate (O6:K2:H31)	[33]
CFT073	Urosepsis isolate (O6:K2:H1)	[95]
UTI89	Cystitis isolate (O18:K1:H7)	[96]
536	Pyelonephritis isolate (O6:K15:H31)	[97]
RS218	Neonatal meningitis isolate (O18ac:K1:H7)	[96, 98]
EC958	UTI isolate (O25b:H4)	[99]
HS	Commensal gut isolate (O9:H4)	[100]
MG1655	K12 lab strain	[101]
NMEC1	Neonatal meningitis isolate	ARUP
NMEC2	Neonatal meningitis isolate	ARUP
BEC1	Blood isolate	ARUP
BEC2	Blood isolate	ARUP
BEC3	Blood isolate	Weyrich lab
BEC4	Blood isolate	Weyrich lab
BEC5	Blood isolate	H. Crandall
BEC6	Blood isolate	H. Crandall
BEC7	Blood isolate	H. Crandall
BEC8	Blood isolate	H. Crandall
BEC9	Blood isolate	H. Crandall
F11/pGEN-GFP(LVA)	Encodes destabilized GFP (half life ≈ 40 min) under control of the *em7* promoter, Amp^R	[28]
F11/pKM208	Encodes IPTG inducible lambda Red recombinase, Amp^R	[87]
F11Δ*cnf1*	F11 *cnf1::kan*	This study

Table 2.3. Oligonucleotides used in Chapter 2.

Primer	Sequence (5'-3')
zEF1α	
Forward	TGAGCGTGGTATCACCATTG
Reverse	CAACACCACCAGCAACAATC
zIL1β	
Forward	TGGACTTCGCAGCACAAAATG
Reverse	CACTTCACGCTCTTGGATGA
zTNFα	
Forward	AAGGAGAGTTGCCTTTACCG
Reverse	ATTGCCCTGGGTCTTATGG
zSAA1	
Forward	GGAACTATGAAGCTGCACAGCGG
Reverse	CCTCTGCGAATGAGACCTTG
zHAMP	
Forward	TGCAGGAGAACCAACATCTG
Reverse	AGCAGTATCCGCAGCCTTTA
zMMP9	
Forward	CATTAAAGATGCCCTGATGTATCCC
Reverse	AGTGGTGGTCCGTGGTTGAG
zIL10	
Forward	TCACGTCATGAACGAGATCC
Reverse	CCTCTTGCATTTCACCATATCC
zIFN1	
Forward	TCTGCGTCTACTTGCGAATG
Reverse	GGCTTGGAAATGGTGTCTCC
zIL8	
Forward	TGTGTTATTGTTTTCCTGGCATTTC
Reverse	GCGACAGCGTGGATCTACAG
zIL17c	
Forward	GTCGGAGAGCAAAGTGGAAG
Reverse	GCAGCCATCACACAAACACT
zIL6	
Forward	TCCTCAAACCTTCAGACCGC
Reverse	TCAGGACGCTGTAGATTCGC
F11Δ*cnf1* Knock Out	
Forward	GATAAGGTGTAGTAAAATATTAATCTTCACAGAGGAGTGTGTAG GCTGGAGCTGCTTCG
Reverse	GGAGTAACTATAACAATGGCCAATAAATAATTTCCCGAACATAT GAATATCCTCCTTAG
F11Δ*cnf1* Confirmation	
Forward	CTCTCGCCCAGTGATTAGGT
Reverse	TTGCGCTAACAAAACAGCAC

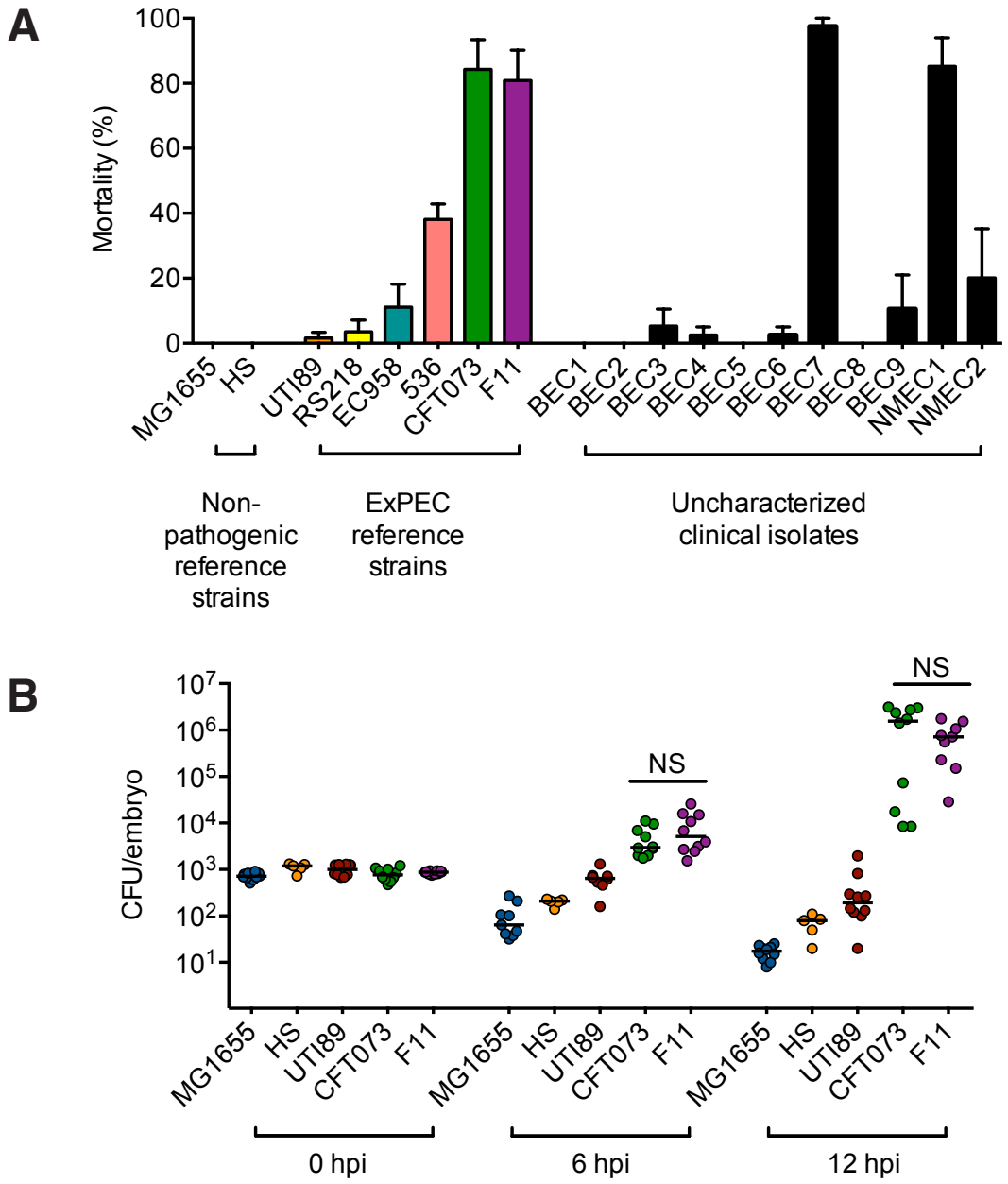

Figure 2.1. Few *E. coli* strains can proliferate and cause death in the bloodstream.

(**A**) Lethality of nonpathogenic reference strains, pathogenic reference strains, and uncharacterized clinical isolates in the zebrafish embryo bloodstream at 24 hpi. Bars show percentage (± SEM) of fish that were killed by ≈1000 CFU of each strain at 24 hpi following inoculation into the bloodstream. n = 30-60 embryos pooled from 2-4 experiments. (**B**) Bacterial burden in infected embryos at 0, 6, and 12 hpi. Each circle represents an individual embryo with data pooled from two independent experiments. Lines mark median values. CFT073 and F11 did not show significantly different titers at any time point, as measured by student's t-test.

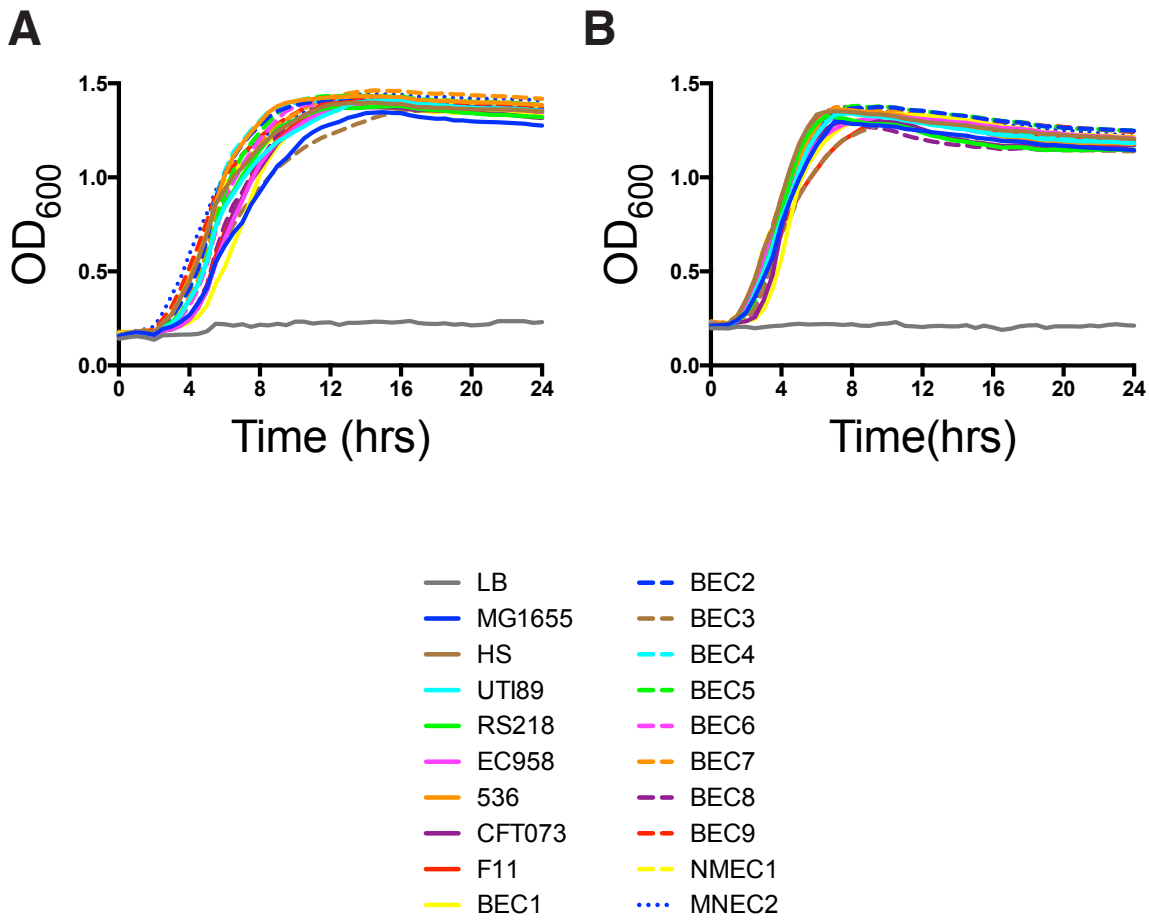

Figure 2.2. Similar growth rates of *E. coli* strains in broth culture.
(**A-B**) Representative data showing that all strains tested share similar growth kinetics in LB broth at 28.5°C (**A**) and 37°C (**B**). Each graph is representative of three independent experiments performed in quadruplicate.

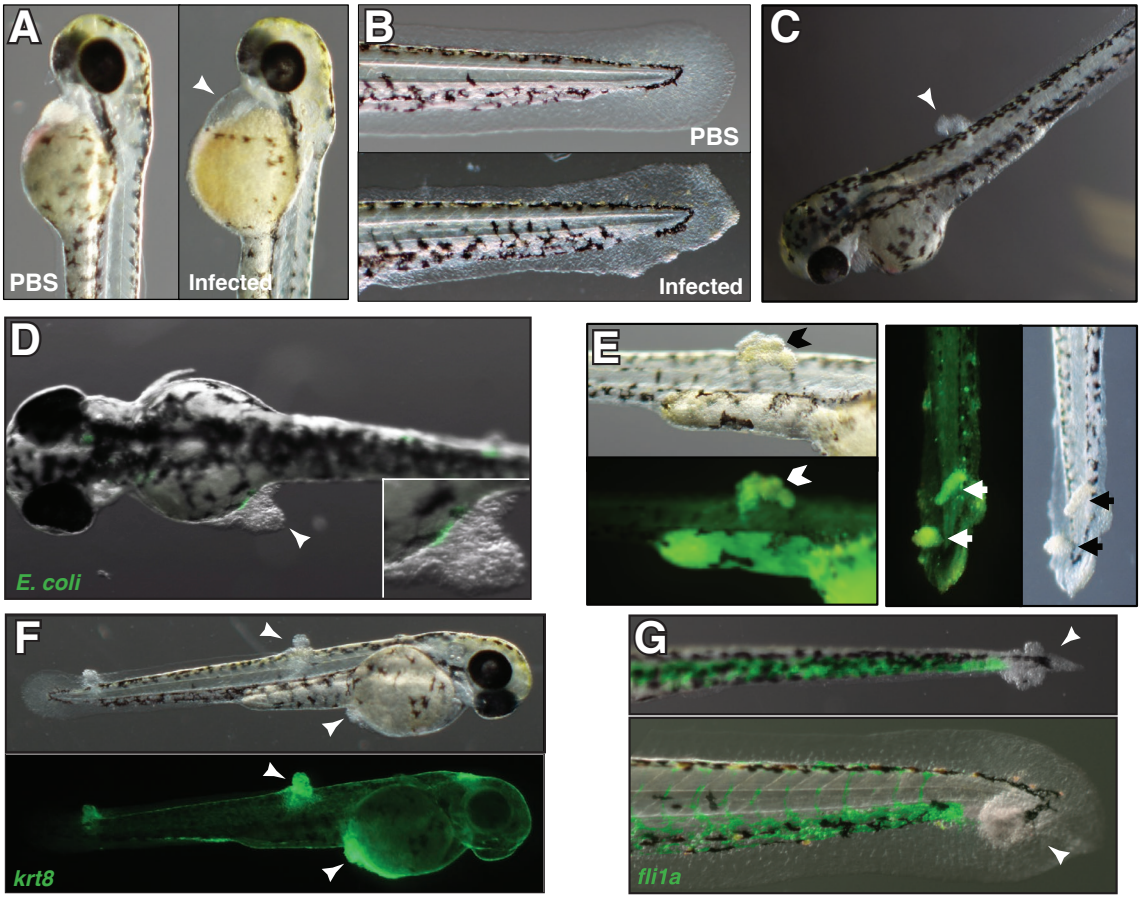

Figure 2.3. Distinct pathologies associated with different, but equally lethal ExPEC isolates.
(**A**) An F11-infected embryo displaying characteristic pericardial edema (arrowhead) at 12 hpi. This is not seen following injection of controls with PBS (left). (**B**) A CFT073-infected embyro at 12 hpi showing fin erosion and ulceration commonly seen during infection with either CFT073 or F11. (**C**) F11-infected embryos often develop protrusions (arrowhead) on the trunk or tail regions by 12 hpi. (**D**) Merged bright field and fluorescent images of an F11/pGEN-GFP(LVA)-infected embryo at 12 hpi showing bacteria at the base of a protrusion (inset), but not within the main structure. (**E**) Bright field and matched fluorescent images of F11-infected embryos stained with acridine orange (green) at 12 hpi. The dye accumulates within protrusions on the trunk (left, chevron) and tail (right, arrows). (**F**) Bright field (top) or fluorescent (bottom) images of Tg(*krt8*:GFP) embryos 12 hpi with F11. Epithelial cells within this transgenic line express GFP. Arrowhead marks an F11-induced protrusion. (**G**) Merged bright field and fluorescent images of Tg(*fli1a*:GFP) embryos at 12 hpi with F11. Endothelial cells and leukocytes in this transgenic line express GFP. Protrusions are indicated with arrowheads.

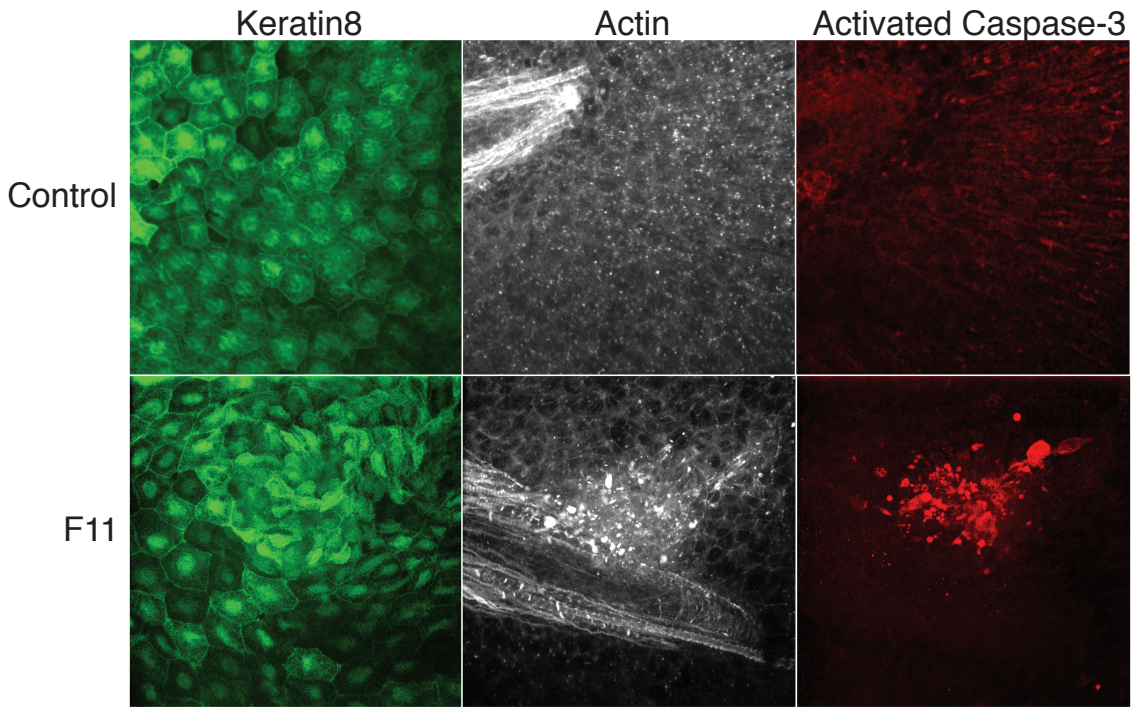

Figure 2.4. Perturbed actin architecture and activated caspase-3 present in tissue lesions.
Fluorescent images of Tg(*krt8*:GFP) embryos infected with F11 at 12 hpi and stained with Phalloidin (middle) and anti-active Caspase-3 (right). Epithelial cells within this transgenic line express GFP (left).

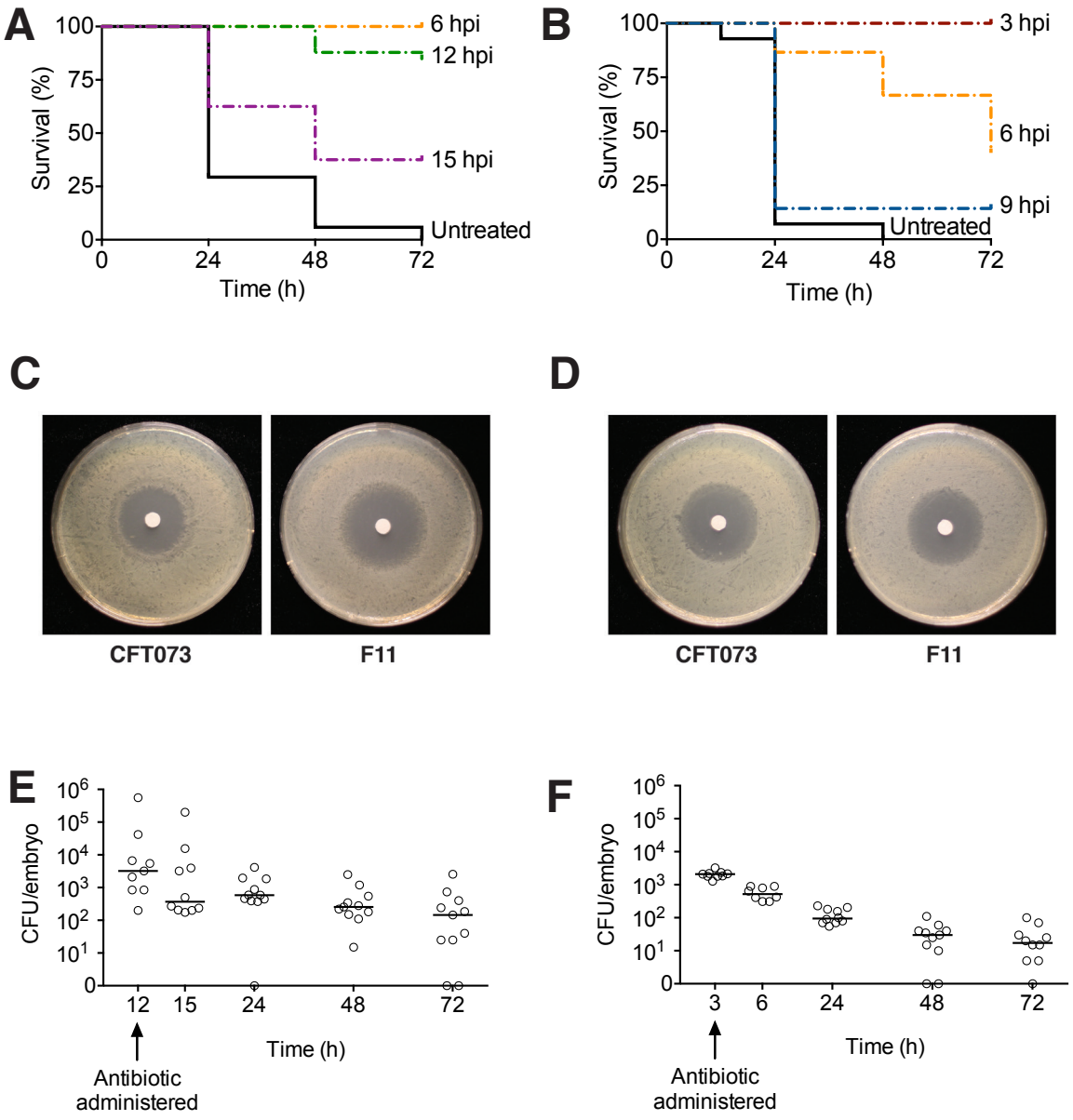

Figure 2.5. Survival of ExPEC-infected embryos varies with ciprofloxacin treatment

(**A-B**) Kaplan-Meier survival curves of zebrafish embryos injected systemically via the circulation valley with (**A**) F11 or (**B**) CFT073 and then treated with ciprofloxacin at the indicated times; n=15-20 embryos. Black lines shows untreated controls

(**C-D**) Representative images of ciprofloxacin disc diffusion assays on LB plates incubated overnight at 28.5°C (**C**) and 37°C (**D**).Each disc contains 5 µg ciprofloxacin.

(**E-F**) Bacterial titers recovered from (**E**) F11- and (**F**) CFT073-infected embryos before and after treatment with ciprofloxacin at 12 or 3 hpi, as indicated. Bars denote median values for each group; n≥9 embryos pooled from two independent experiments.

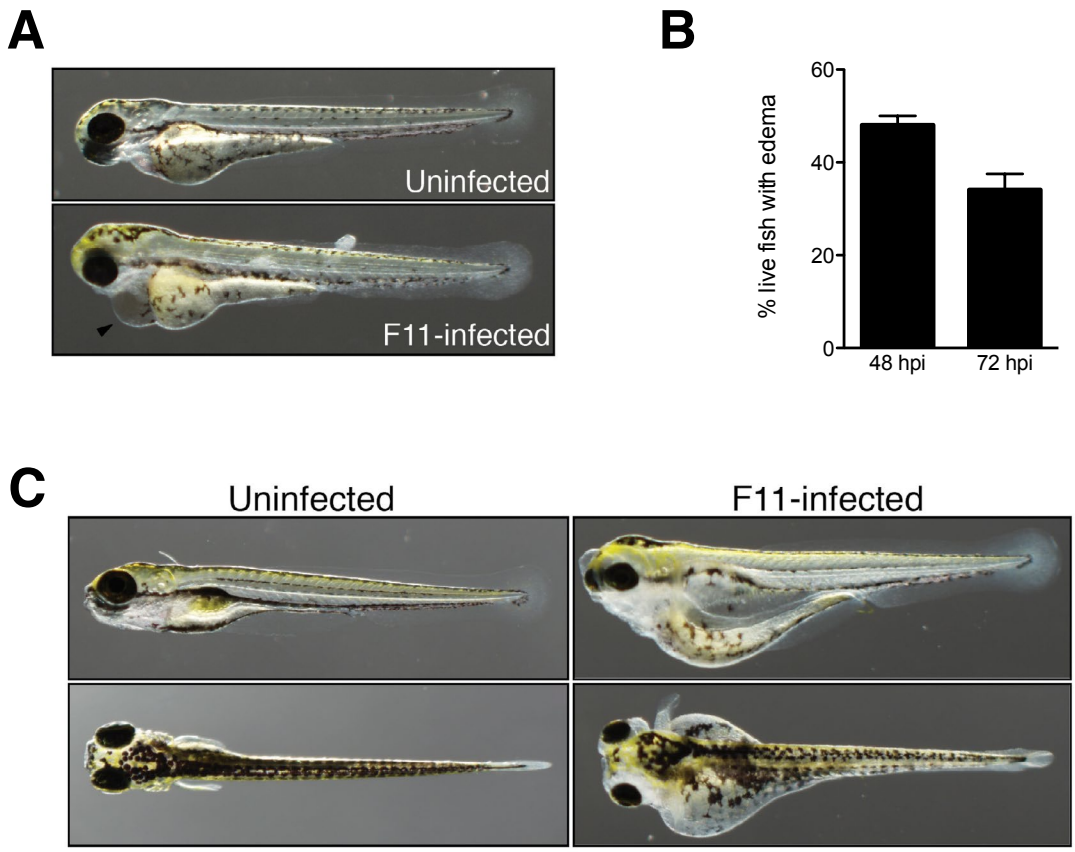

Figure 2.6. Infected embryos develop profound edema following rescue with ciprofloxacin.
(**A and C**) Images show uninfected control and F11-infected embryos at (**A**) 24 and (**C**) 72 hpi. Both control and F11-infected zebrafish were treated with ciprofloxacin beginning at 12 hpi. Arrowhead in (**A**) indicates the presence of pericardial edema in a surviving F11-infected embryo. (**B**) Percent of surviving embryos with overt signs of edema at 48 and 72 hpi of the bloodstream with F11. Ciprofloxacin was administered at 12 hpi. Bars represent mean data ± SEM from 2 independent experiments; n=30-40 fish total.

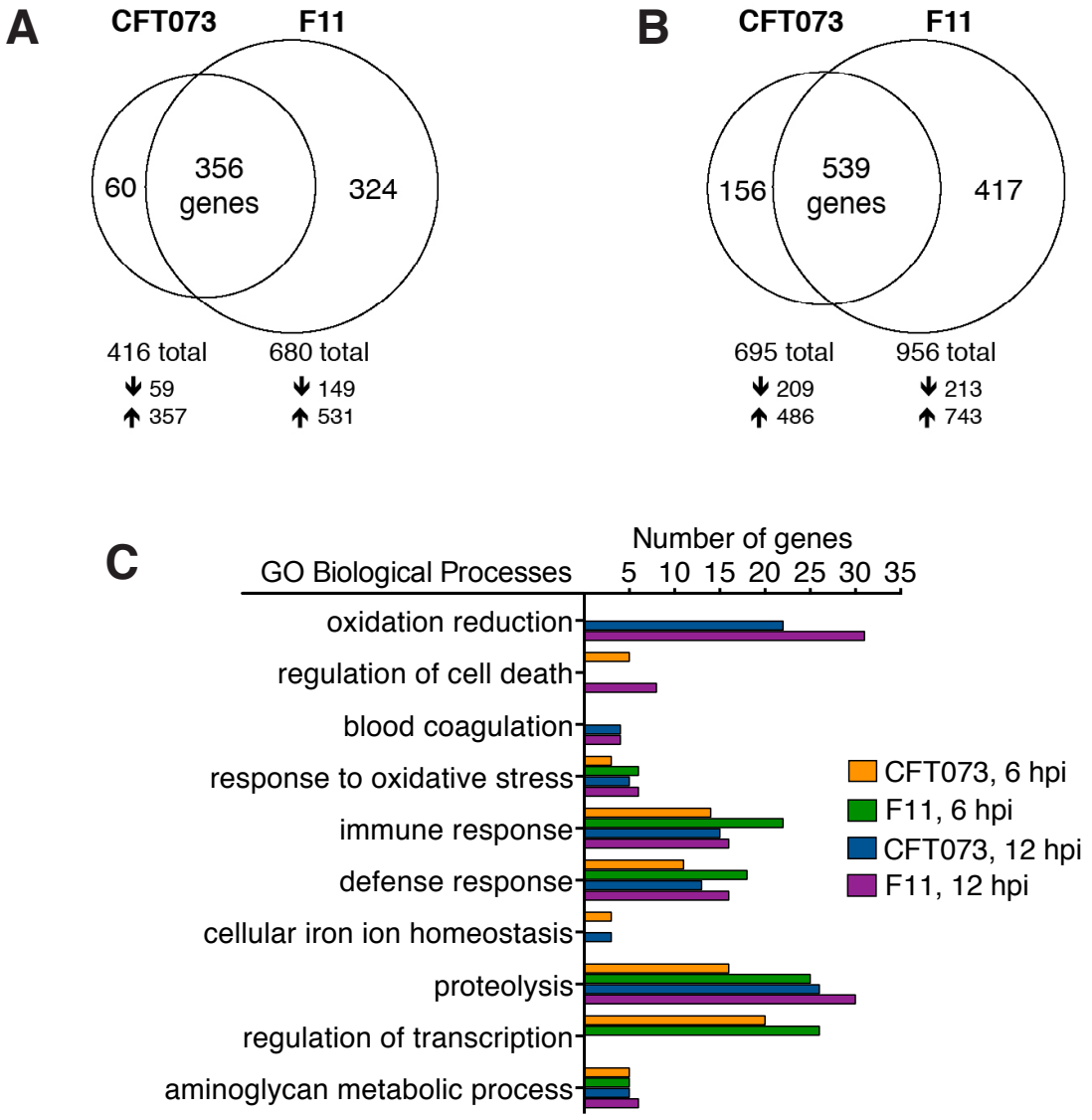

Figure 2.7. Equally lethal ExPEC isolates trigger distinct host responses. (**A-B**) Venn diagrams indicating the numbers of host genes that are differentially expressed in zebrafish embryos at (**A**) 6 hpi or (**B**) 12 hpi with CFT073 versus F11, as determined by microarray analysis. The number of differentially expressed transcripts was calculated for each dataset by gating on probes with ≥2-fold changes relative to mock-infected controls and $P \leq 0.05$. Results from multiple probes that mapped to the same gene were combined to generate non-redundant lists of the differentially expressed genes. (**C**) The number of differentially expressed host genes in a selected list of enriched GO terms, as determined by DAVID.

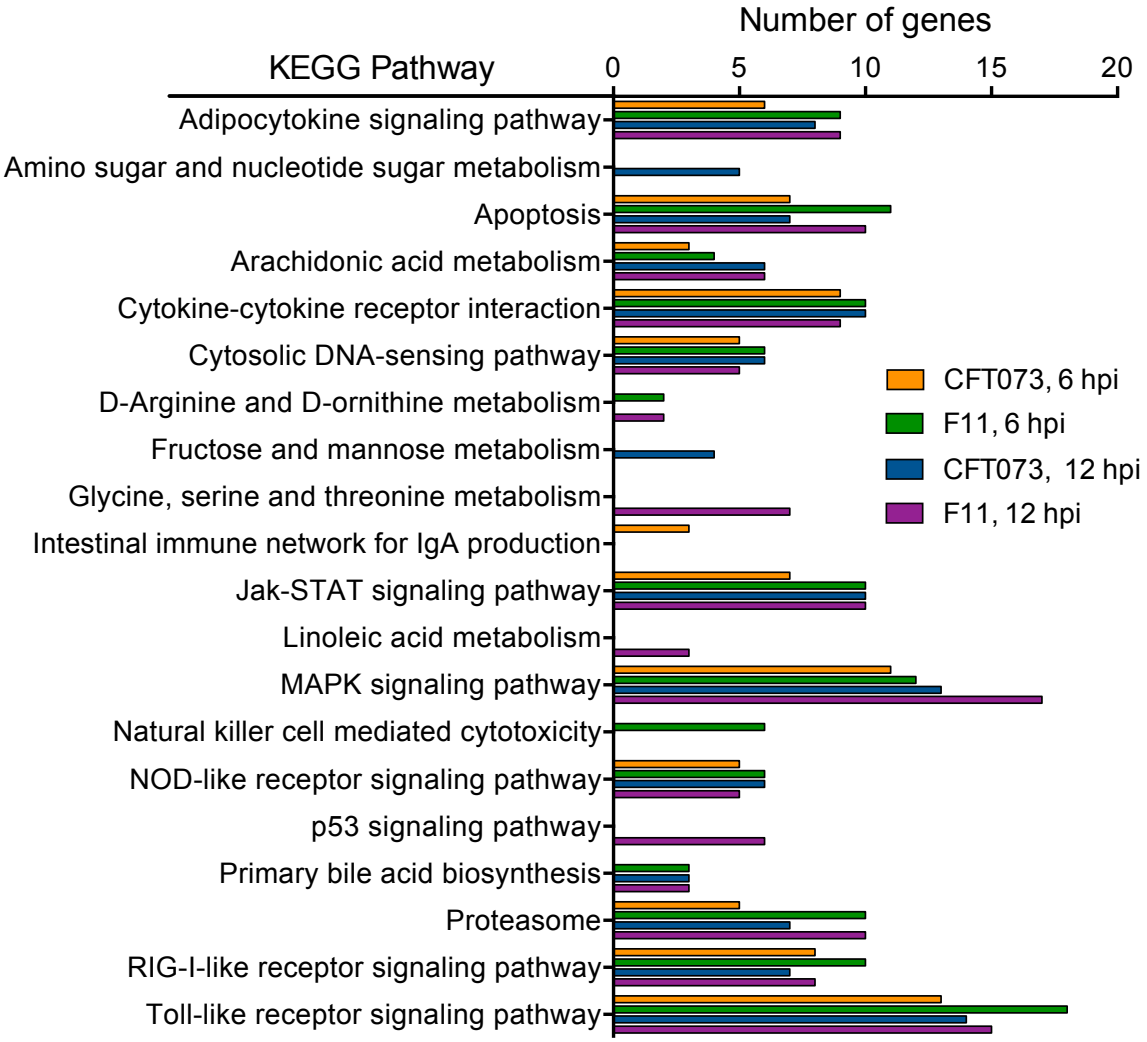

Figure 2.8. CFT073 and F11 have differential effects on specific host pathways.

Quantification of differentially expressed genes (fold change ≥2, $P \leq 0.05$) within KEGG pathways that are affected in response to systemic infections of zebrafish embryos with CFT073 and/or F11, as determined by DAVID.

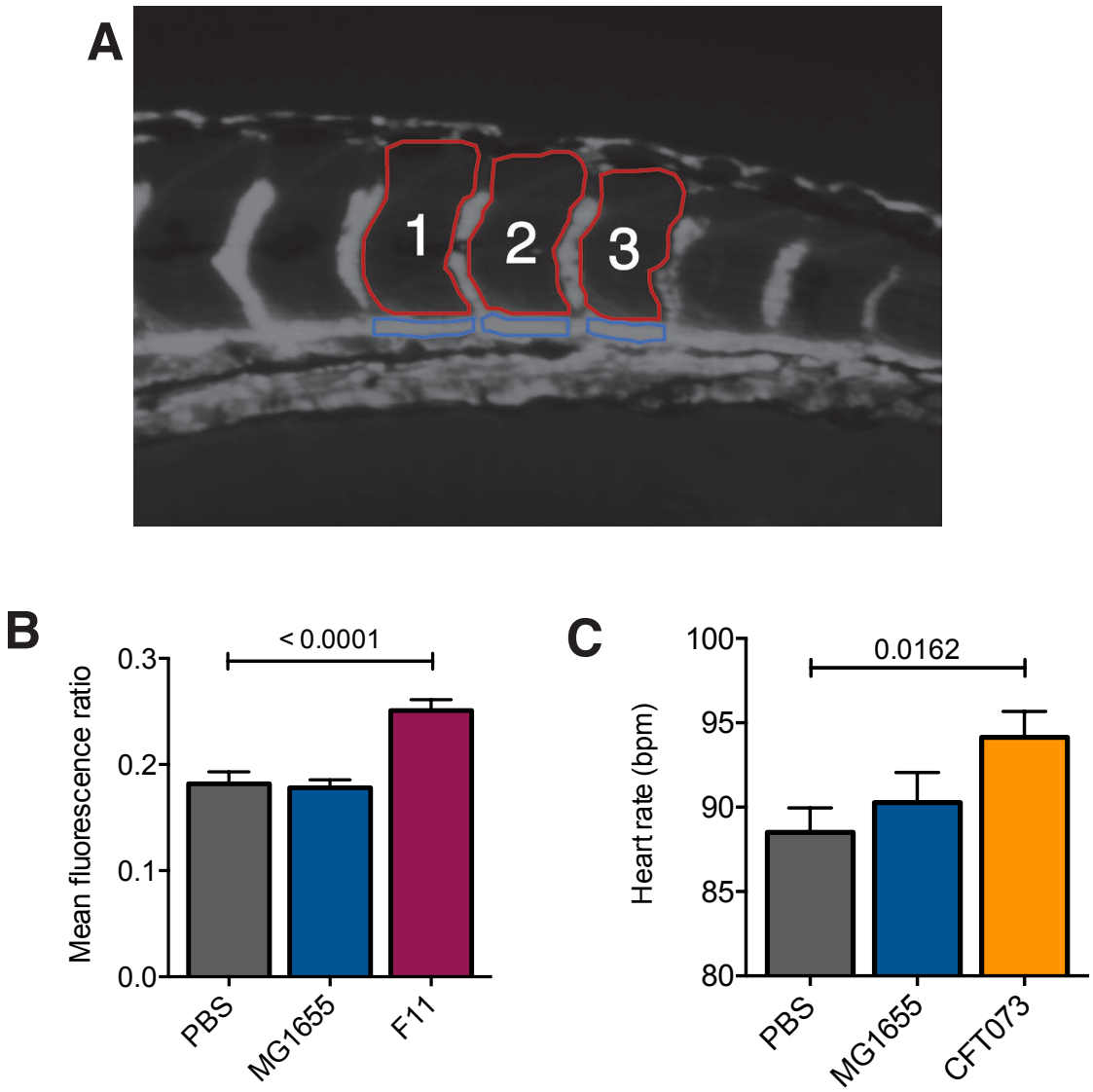

Figure 2.9. ExPEC induces tachycardia and vascular leakage in zebrafish embryos.

(**A**) Image shows the tail region of an embryo following intravenous injection of fluorescent 70-kDa dextran. Using ImageJ, the mean fluorescent intensities of 3 specific myotomes (red outlines) and the sections of caudal artery below each myotome (blue outlines) were measured. Dextran leakage was quantified by calculating the ratio of fluorescent intensity within the myotomes to the underlying vasculature.

(**B**) Endothelial leakage in infected and control PBS-injected embryos at 9 hpi. Data indicate mean values ± SEM from 2 independent experiments; n=9-15 embryos with 3 measurement sites used per fish.

(**C**) Heart rate (beats/min) in infected and PBS-injected control zebrafish embryos at 6 hpi. Each bar represents mean values ± SEM from 2 independent experiments; n=10-12 embryos. The indicated P values were determined by unpaired Student's t tests.

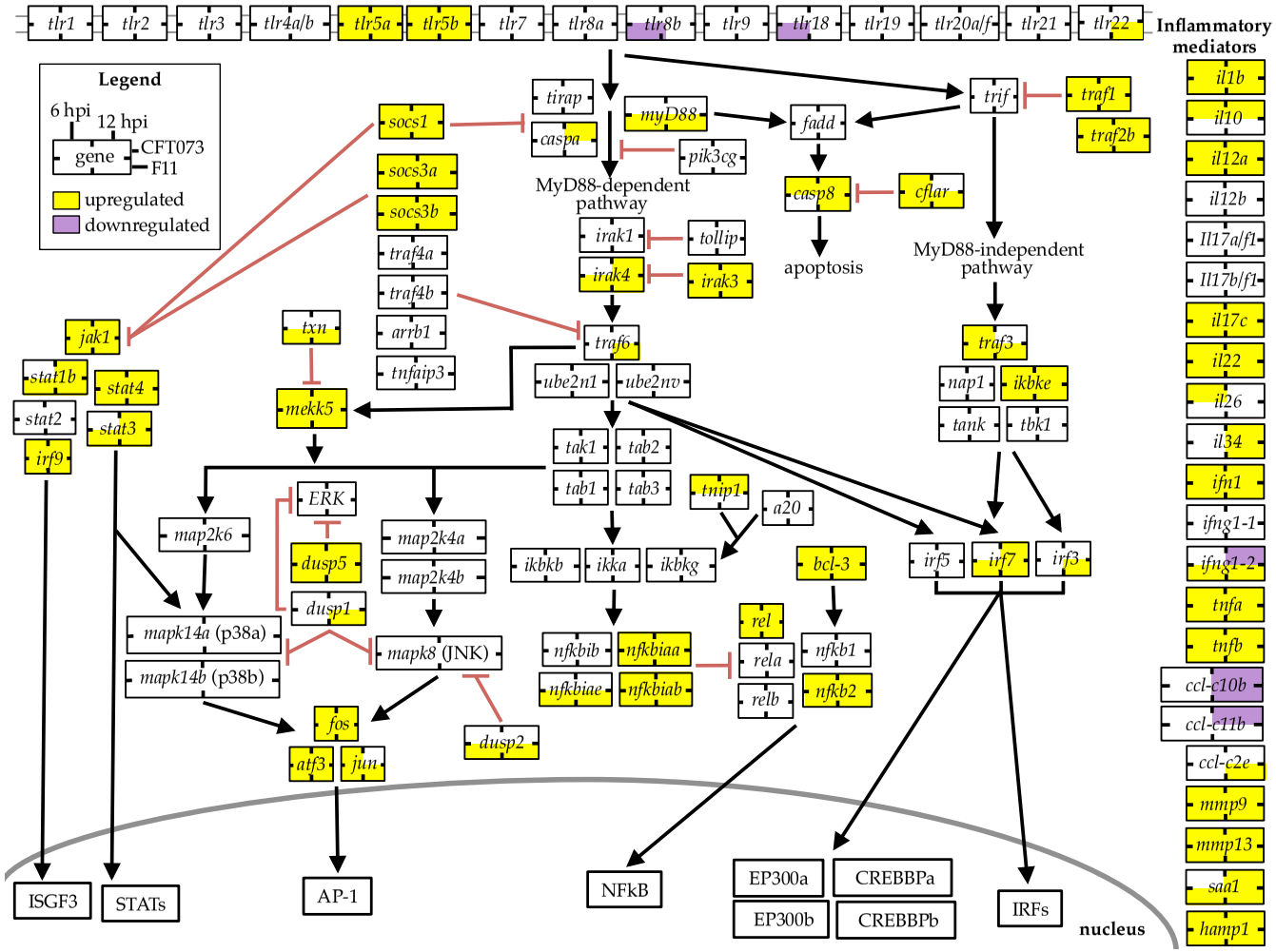

Figure 2.10. CFT073 and F11 have overlapping, but distinct, effects on the expression of TLR pathway genes and downstream inflammatory mediators.

Diagram shows differentially expressed host genes at 6 and 12 hpi with CFT073 or F11. Upregulated genes are denoted in yellow, while those that are downregulated are purple (fold change up or down ≥2.0; p≤0.05).

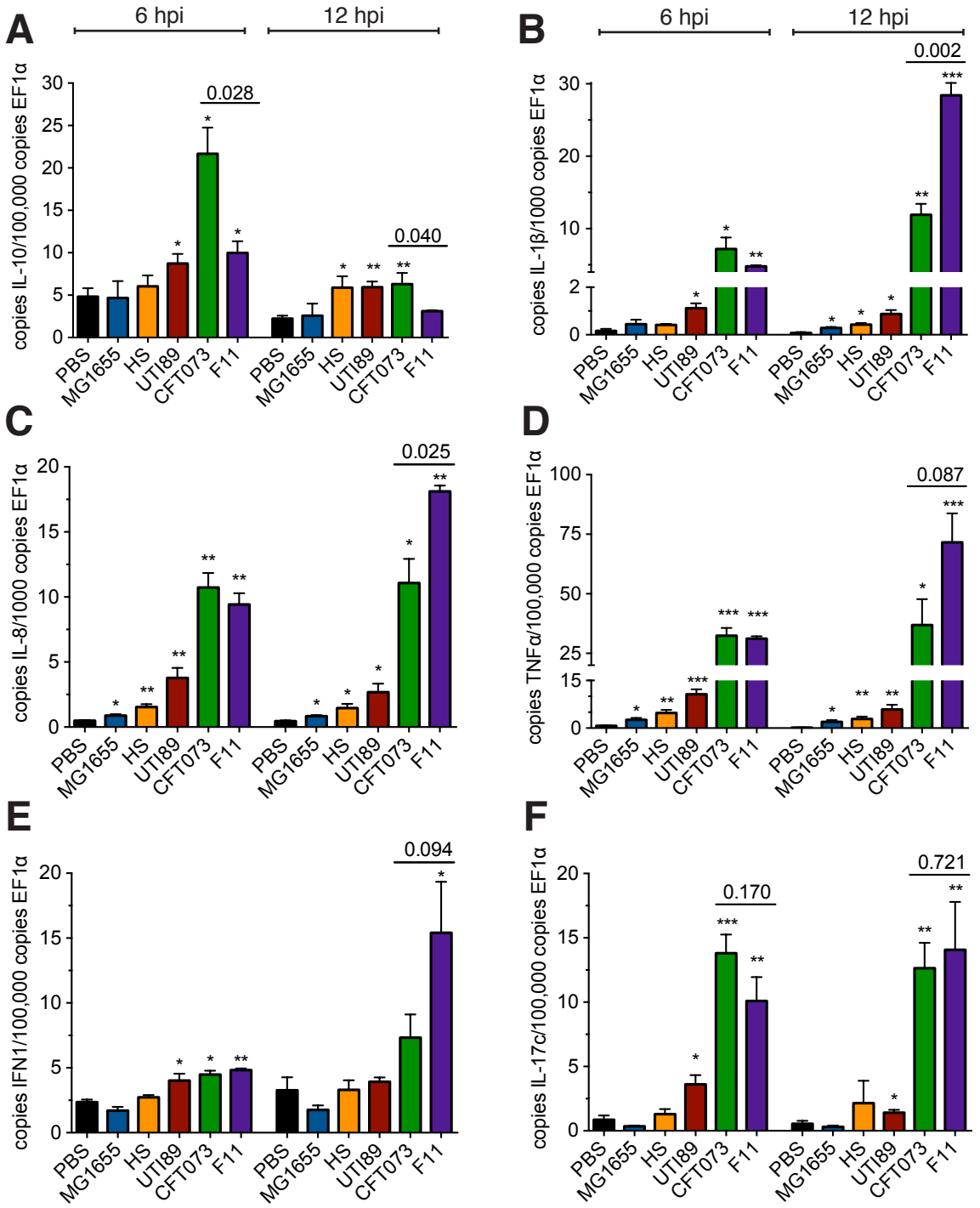

Figure 2.11. Variable expression of cytokines in response to lethal and nonlethal *E. coli* strains.

(**A-G**) qRT-PCR analysis of the specified cytokine transcripts at 6 and 12 hpi with PBS or ~1,000 CFU of the indicated *E. coli* strains. Each bar represents mean results ± SEM from 3-4 pools of ≥16-20 embryos. qRT-PCR for each pool was carried out using technical duplicates. **P*≤0.05, ***P*≤0.01, ****P*≤0.001 versus PBS-injected controls, as determined by Student's t tests. *P* values for F11- versus CFT073-infected samples are also indicated.

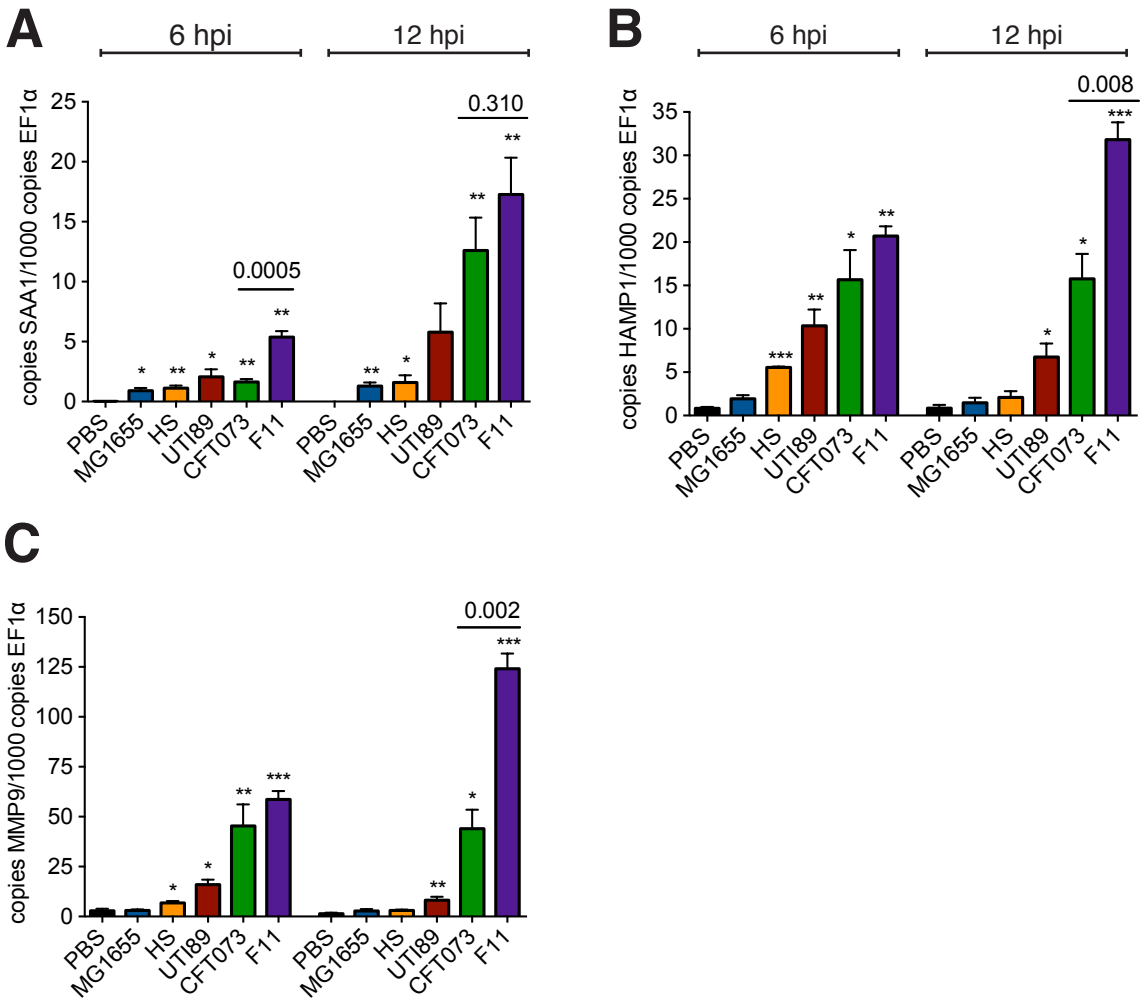

Figure 2.12. Variable expression of host defense genes in response to lethal and nonlethal *E. coli* strains.

(**A-C**) qRT-PCR analysis of the specified cytokine transcripts at 6 and 12 hpi with PBS or ~1,000 CFU of the indicated E. coli strains. Each bar represents mean results ± SEM from 3-4 pools of ≥16-20 embryos. qRT-PCR for each pool was carried out using technical duplicates. **P*≤0.05, ***P*≤0.01, ****P*≤0.001 versus PBS-injected controls, as determined by Student's t tests. *P* values for F11- versus CFT073-infected samples are also indicated.

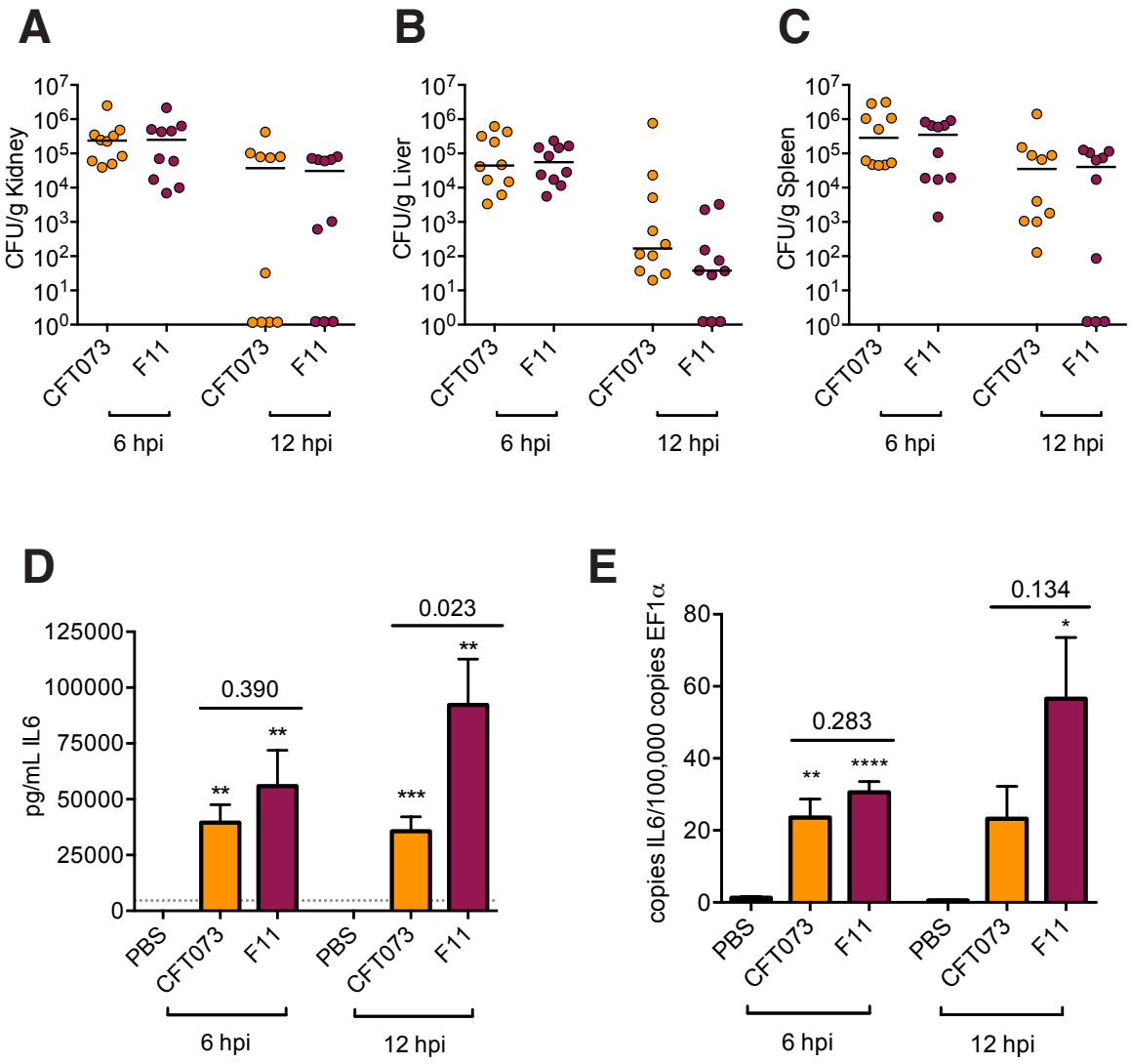

Figure 2.13. ExPEC strain differences observed in the zebrafish are recapitulated in a mouse model of sepsis.

(**A-C**) Bacterial titers per gram organ in the kidney (**A**), liver (**B**), and spleen (**C**) of CFT073- and F11-infected mice at 6 and 12 hpi. Each circle represents an individual mouse with data pooled from two independent experiments (n = 10 mice total for each time point). Lines mark median values. CFT073 and F11 did not show significantly different titers in any organ at any time point as measured by student's t-test.

(**D**) Serum IL-6 levels during sepsis. Each bar represents the mean ± SEM with 5 mice per group and time point. Gray line is the limit of quantification. P = *≤0.05, **≤0.01, ***≤0.001, **** ≤0.0001 when compared to PBS-injected controls.

(**E**) IL-6 transcript levels of fish injected with ≈1000 CFU CFT073 or F11, or a similar volume of sterile PBS as a control. Each bar represents the mean (± SEM) of 3 pools of 15-20 embryos. qRT-PCR was done in technical duplicate.

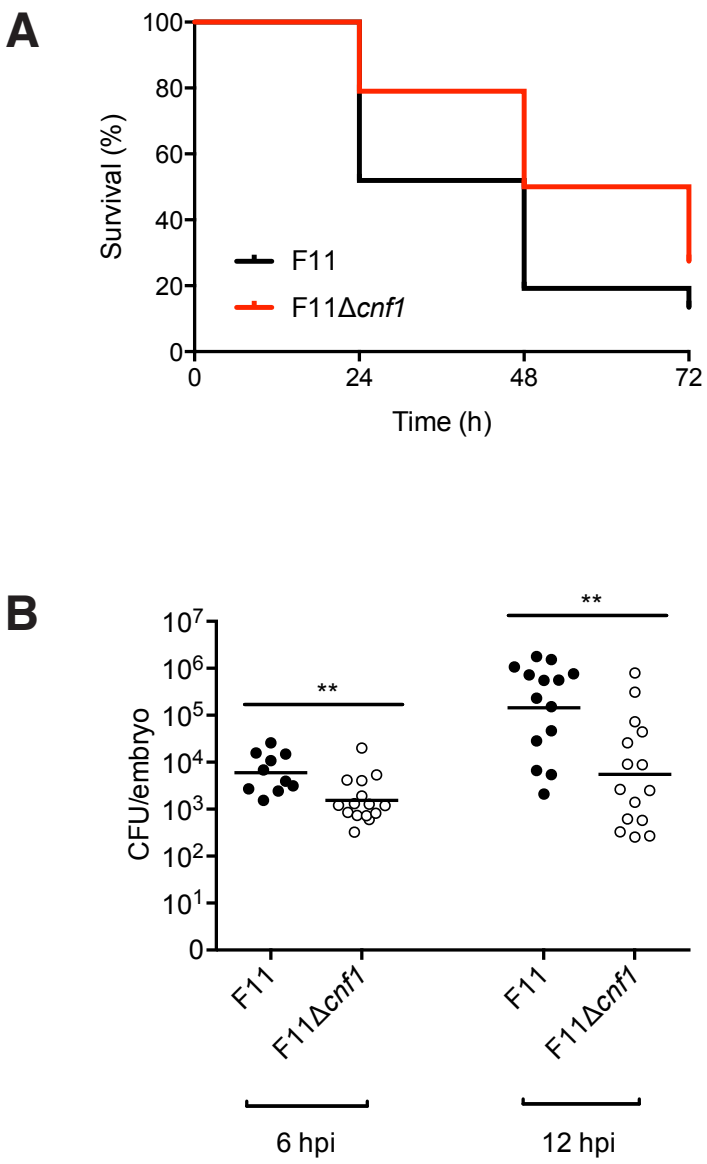

Figure 2.14. CNF1 is an important virulence determinant in the zebrafish bloodstream.

(**A**) Kaplan-Meier survival curves of zebrafish embryos injected via the circulation valley with 1000-1800 CFU of F11 or F11Δ*cnf1*; n≥51 embryos pooled from two independent experiments. Curves are significantly different (p=0.0018) by Mantel-Cox log-rank test.

(**B**) Bacterial titers recovered from F11- or F11Δ*cnf1*-infected embryos. Bars denote median values; n≥11 embryos pooled from two independent experiments. **p≤0.01, as determined by Mann-Whitney U tests.

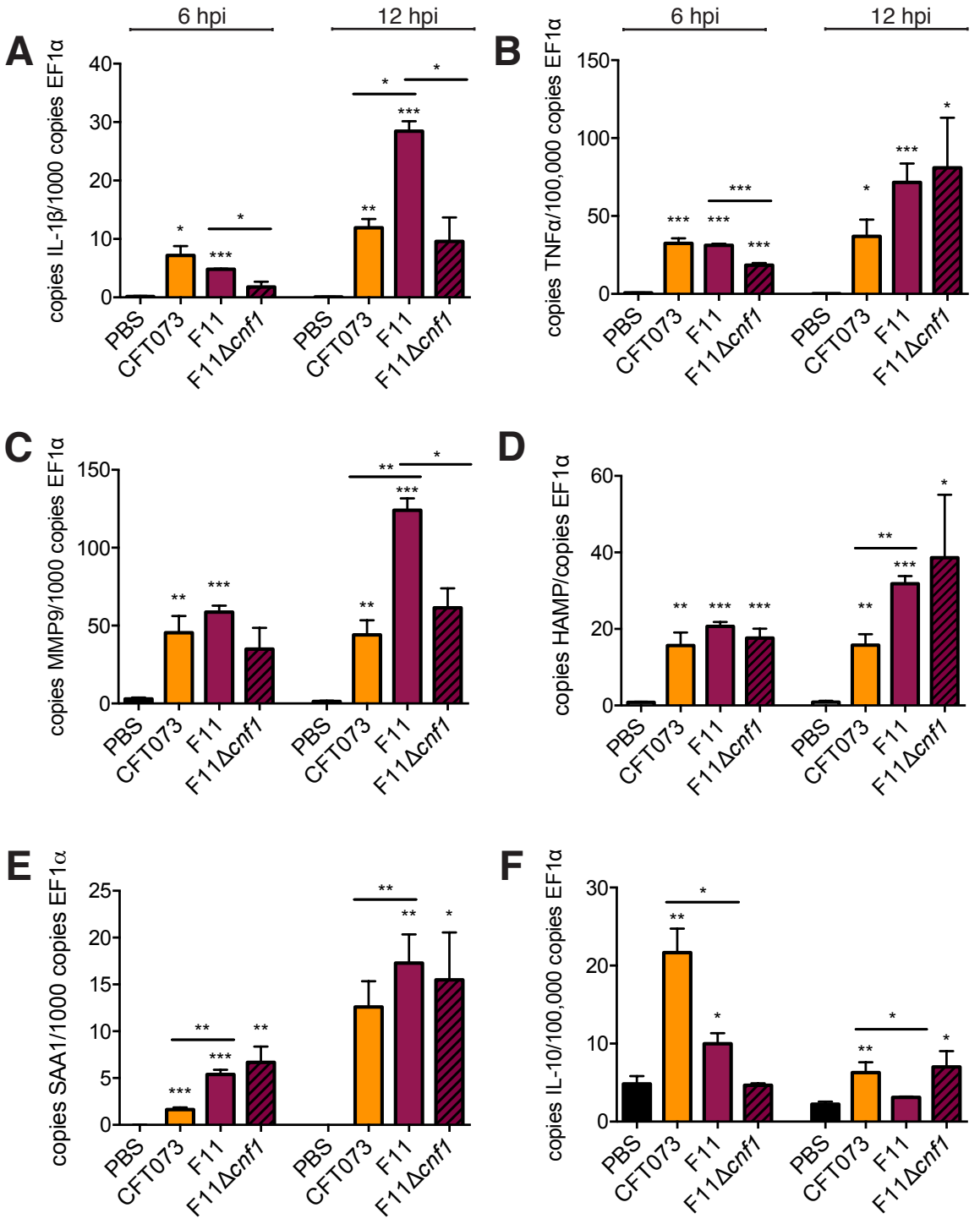

Figure 2.15. Variable cytokine expression in response to CFT073, F11, and F11Δ*flic*.

(**A-G**) qRT-PCR analysis of the specified cytokine transcripts at 6 and 12 hpi with PBS or ~1,000 CFU of the indicated *E. coli* strains. Each bar represents mean results ± SEM from 3-4 pools of ≥16-20 embryos. qRT-PCR for each pool was carried out using technical duplicates. **P*≤0.05, ***P*≤0.01, ****P*≤0.001 versus PBS-injected controls or strain indicated, as determined by Student's t tests.

CHAPTER 3

BACTERIAL FLAGELLIN IS A KEY MODULATOR OF INFLAMMATION DURING *ESCHERICHIA COLI* SEPSIS

Abstract

Sepsis is the systemic and life-threatening activation of the immune system in response to invading microorganisms. *Escherichia coli,* the most common cause of bloodstream infections and second leading cause of sepsis, is of great concern clinically given the increasing incidence of multidrug resistant strains. Previous work has established that genetically distinct *E. coli* isolates CFT073 and F11 induce divergent host responses during sepsis, independent of baseline host attributes, offering a partial explanation for the variability in disease progression and response to treatment seen in septic individuals. Using a zebrafish model of sepsis, I show that these disparate host responses are the result of differences in the levels of bacterial flagellin expressed by CFT073 and F11 during sepsis, as well as in the genetically distinct flagellin variants produced by each strain differentially activate TLR5. I also present a novel system in which recombinant flagellin is produced by mammalian cells in culture, in the absence of any other bacterial products, to define the contributions of candidate residues and domains within flagellin to TLR5 activation.

Introduction

Sepsis is an acute systemic inflammatory condition marked by high mortality rates ranging from 17-49%. Compounding the problem of sepsis is its high degree of patient heterogeneity, making it notoriously difficult to diagnose and manage effectively [1, 2]. The incidence of sepsis is also on the rise, with hospitalization rates doubling since 2000 and associated costs reaching greater

than $20 billion annually, putting a significant financial burden on the healthcare system [3]. *E. coli* are the principal cause of bacteremia and a leading cause of sepsis, second only to Group B *Streptococcus* in neonates and *Staphylococcus aureus* in adults [4-8]. Sepsis caused by *E. coli* is more likely to cause death compared to these Gram-positive pathogens [9, 10]. The rate of multidrug-resistant *E. coli* has increased in recent years, greatly limiting the effectiveness of current antibiotic therapies [6, 11, 12].

Much of the variability in disease progression in septic patients has been attributed to differences in the genetic background and immune status of individual hosts [13-15]. However I demonstrate in Chapter 2 that lethal but genetically distinct *E. coli* strains are also capable of contributing to differences in host responses, particularly in TLR signaling pathway genes, independent of host background. However, the underlying bacterial cause of these divergent host responses remains unknown.

Flagella are best known as the locomotor organelles involved in bacterial motility and chemotaxis [16]. However flagellin, the ligand of TLR5 and the principal component of bacterial flagella, is also a potent inducer of inflammation and well-established virulence factor for *E. coli* and other pathogens [16-21]. Flagellin protein is composed of highly conserved N- and C- termini and a hypervariable center domain. TLR5 binding and signaling are mediated through the conserved regions, while the hypervariable domain is canonically dispensable for TLR5 activation but responsible for generating serotype diversity [22-24]. The

contributions of serotype variability to pathogenesis have historically only been studied in the context of its importance for antibody-mediate immune evasion. However there is emerging evidence that flagellin (FliC) variants can differentially modulate TLR5 activation and cytokine production [25-30]. There are currently 56 characterized H (flagellar) antigens in *E. coli* [31], but the contributions of these different variants to TLR5 activation and downstream cytokine production remain largely unknown.

In this chapter I establish that for two lethal ExPEC strains, CFT073 and F11, the observed differences in host response during sepsis are the result of variable flagellin expression as well as how genetically distinct flagellin molecules differentially activate TLR5, unappreciated phenomenon that could account for the variability in symptoms observed in human septic patients. I also present a novel system in which recombinant flagellin protein is produced by mammalian cells in culture in the absence of any other bacterial products.

Results

Flagellin from CFT073 and F11 differentially modulate inflammatory responses during sepsis

CFT073 and F11 cause different host responses (Figures 2.7-2.8, 2.10-2.13). However the cause is unknown. CFT073 and F11 share the same type 2 (K2) capsule and O6 LPS antigens, but possess distinct flagellar serotypes (H1 vs. H31, respectively). The flagellin proteins from CFT073 and F11 are only 67.3% similar at the protein coding level, with each being comprised of well-

conserved N- and C-terminal domains separated by a variable middle domain that is responsible for generating serotype diversity (Figure 3.1). This domain architecture is common among bacterial flagellin proteins and provides the basis for flagellin recognition by TLR5 [16, 22, 24]. Regions within the conserved termini of FliC that are predicted to be bound by TLR5 are nearly identical in the CFT073 and F11 sequences, with the exception of a single conservative isoleucine to valine change.

Considering the stimulatory effects of both CFT073 and F11 on the transcription of TLR5 and associated signaling factors (see Figures 2.8, 2.10, and 2.11), I wished to define the contributions of the FliC variants to the infection process and host responses. Deletion of *fliC* in either CFT073 or F11 rendered both pathogens immobile on swim agar plates (Figure 3.2), but had no effect on bacterial titers recovered from the zebrafish host at 12 hpi (Figure 3.3A). At this time point CFT073Δ*fliC* induced similar levels of the pro-inflammatory cytokines IL-1β and IL-8 as its wild type counterpart, whereas F11Δ*fliC* was markedly less inflammatory than the wild type F11 strain (Figure 3.3B and C).

The levels of flagellin expression by CFT073 and F11 during infection of the zebrafish host were examined using a low-copy reporter construct in which the *luxCDABE* gene cluster encoding bacterial luciferase is transcriptionally fused with the conserved *fliC* promoter (p_{fliC}-*lux*) [18]. Titers of the recombinant strains present at 12 hpi were comparable, though marginally fewer F11/p*fliC*-lux were recovered (Figure 3.4A). Despite this discrepancy, significantly higher levels of

fliC expression were detected in zebrafish infected with F11/p*fliC*-lux versus CFT073/p*fliC*-lux (Figure 3.4B). Disparity in the observed host responses to F11 and CFT073 correlates with variations in the levels of flagellar expression by these two ExPEC isolates.

In addition, using a TLR5 reporter cell line (HEK-Blue mTLR5 cells), I found that semipurified preparations of flagella from F11 were significantly more stimulatory than preparations from CFT073 (Figure 3.4C), despite the presence of similar amounts of FliC (as verified by immunoblot, Figure 3.4D). In these assays, HEK-Blue mTLR5 cells treated with preparations from CFT073Δ*fliC* or F11Δ*fliC* showed no significant activation above baseline. Further confirming the specificity of the measured responses, I found that the addition of anti-TLR5 neutralizing antibody, but not an isotype control, significantly inhibited stimulation of the TLR5 reporter cells by flagella from both CFT073 and F11 (Figure 3.4E). In total, these data indicate that the divergent inflammatory responses seen in F11- and CFT073-infected zebrafish are in part attributable to differences in the levels of FliC expression by these two pathogens as well as contrasting stimulatory effects of the two FliC variants on TLR5 signaling.

Knockdown of TLR5 increases bacterial burden during sepsis, but does not alter cytokine levels

To directly examine the contributions of TLR5 to host response and disease outcome during sepsis, I used morpholinos (antisense oligonucelotides) against *tlr5a* and *tlr5b* to reduce levels of these genes in the zebrafish.

Knockdown of zTLR5a/b did not significantly alter the survival rate of infection with CFT073 or F11 when compared to larvae injected with a control morpholino (Figure 3.5A). However, TLR5 morphants showed a significantly increased bacterial burden at 12 hpi during infection with CFT073 and F11 compared to control morphants (Figure 3.5B). Despite the increased bacterial load, the abrogation of TLR5 did not significantly alter levels of downstream inflammatory mediators as similar amounts of *il1β* (Figure 3.5C) or *il8* (Figure 3.4D) were found between control and TLR5 morphants at 12 hpi during infection with CFT073 and F11.

Bacterial-free protein expression system for the identification of residues and domains that contribute to differential stimulation of TLR5

The predicted canonical TLR5-binding sites within the FliC variants from CFT073 and F11 are nearly identical, save for one residue (isoleucine 528 in CFT073 and valine 488 in F11) that sits on the backside of the C-terminal α-helix that interfaces with TLR5 [22, 24]. This change, as well as multiple synonymous SNPs present within coding sequences for the conserved termini of the FliC variants could potentially affect signaling via TLR5. Though the center hypervariable domain of FliC does not make direct contact with TLR5, previous studies indicate that this domain may also modulate cytokine responses [27, 32]. To determine the contributions of candidate residues and domains to TLR5 signaling by FliC variants from CFT073 and F11, I created constructs encoding

the full length version of each variant (pBF16 and pBF12 for CFT073 FliC and F11 FliC, respectively), the hypervariable domain alone from each variant [pAB1 for CFT073 FliC(176-497) and pAB2 for F11 FliC(176-466)], and mutant FliC variants where the nonidentical residue in the predicted C-terminal TLR5 binding domain is modified to the residue present in the other variant [pBF16(I528V) and pBF16(V488I)] (Figure 3.6A). With the aim of creating recombinant protein free from contamination of other bacterial products, each of these constructs was cloned into a mammalian expression vector in between an insulin secretory signal and C-terminal 6xhis tag (Figure 3.6B). Following transfection of HEK293T cells, media containing recombinant flagellin was collected and added to a TLR5 reporter cell line. Media from pBF12- and pBF16-transfected cells induced robust activation of TLR5, while no significant activation was seen following treatment with PBS or media from empty vector control cells (Figure 3.6C). Subsequent immunoblot of media from transfected cells indicated that there was substantially more recombinant flagellin in the pBF16-transfected media than the pB12-transfected media, explaining the differential levels of TLR5 stimulation seen between these samples and indicating that activation by recombinant flagellin was dose dependent (Figure 3.6D).

Discussion

Septic patients often present with wide-ranging disease signs and pathophysiological manifestations, which makes diagnosis difficult, and are notoriously difficult to manage because they do not respond homogenously to

treatment [33]. During sepsis, the host background is often considered the primary variable affecting disease progression and outcome, while microbes are commonly viewed as generic inducers of inflammation. In Chapter 2 I demonstrated that *E. coli* isolates belonging to the same species can markedly affect the host response during sepsis, irrespective of host background characteristics. Continuing on this work, here I establish variable expression of bacterial flagellin during sepsis and differential stimulation of the host receptor TLR5 as key factors contributing to differences in host response during sepsis.

The involvement of flagellin as a regulator of host responses during sepsis has precedence. Flagellin can be detected in the plasma of septic patients at concentrations ranging from 2 to 20 ng/mL, with higher levels being associated with longer duration of septic shock [34]. In mouse burn wound models of sepsis, the administration of flagellin can enhance the antibacterial activities of neutrophils and the delivery of antiflagellin antibodies can promote host survival [35, 36]. In both our zebrafish model and in human patients with sepsis-like syndromes, the flagellin receptor TLR5 is among the most highly induced pattern recognition receptors [37-39]. Allelic variants of TLR5 may predispose infants to sepsis, and high-level expression of TLR5 in septic individuals is positively linked with more severe disease [39-41].

The failure of morpholinos targeting *tlr5a/b* to alter cytokine levels in the zebrafish sepsis model may be attributable to technical limitations, rather than TLR5 not being involved in the host response during sepsis. For our experiments,

we used previously published oligonucleotide sequences against TLR5a/b [42, 43], but found significant toxic side effects at the published dose required for knockdown. Some of these effects were also seen in embryos injected with a comparable dose of the standard control morpholino, indicating that they are non-specific. Additionally, targeted knockdown by morpholinos frequently fails to recapitulate mutant phenotypes [44] so to truly determine the contributions of TLR5 to sepsis in our system a genomic knockout of TLR5a/b would be needed. Finally, interpreting data regarding cytokine levels can be somewhat ambiguous in cases where there is an accompanying change in bacterial burden (Figure 3.4C-D).

The capacity of distinct flagellar serotypes to differentially activate TLR5, as demonstrated in this study, indicates an unanticipated mechanism by which different ExPEC isolates may elicit variable inflammatory responses in septic individuals. The predicted canonical TLR5-binding sites within the FliC variants from CFT073 and F11 are nearly identical, save for one residue (isoleucine in CFT073 and valine in F11) that sits on the backside of the C-terminal α-helix that interfaces with TLR5 [22, 24]. This change, as well as multiple synonymous SNPs present within coding sequences for the conserved termini of the FliC variants could potentially affect signaling via TLR5. Though the hypervariable middle domain of FliC does not make direct contact with TLR5, previous studies indicate that this domain may also modulate cytokine responses [27, 32]. The ability of different versions of FliC to elicit contrasting host responses suggests

that some bacteria may utilize flagella as immunomodulators, expanding their function beyond motility.

The ability to purify recombinant proteins in a system free of contamination from bacterial products that stimulate host pathogen recognition receptors is a valuable implement. There have been many instances where LPS contamination is the actual cause of a phenotype, rather than the recombinant molecule being tested [discussed in 45]. The protein purification strategy discussed in this chapter is particularly advantageous for the generation of recombinant eukaryotic protein, which can be inactive when produced in bacteria due to differing or missing post-translational modifications [46]. Conversely, recombinant bacterial proteins generated in our system are likely to have eukaryotic post-translational marks, such as N- or O-linked glycosylations, which would not be present if they were synthesized under native bacterial conditions. Not surprisingly, flagellin generated from HEK293T cells runs larger than flagellin isolated from bacteria on SDS-PAGE, likely as a result of modifications that are not present under native conditions. However, mammalian cell-generated flagellin is still very stimulatory to TLR5, indicating that functionality of the protein is retained. I did not have a chance to test the constructs created to determine the residues and domains that contribute to differential stimulation of TLR5 by the FliC variants from CFT073 and F11 (Figure 3.6A), but elucidating the underlying mechanism would form a foundation by which specific FliC variants could be engineered as regulators of host inflammatory responses with possible therapeutic value.

Materials and Methods

Ethics statement

Animals used in this study were handled in accordance with University of Utah and IACUC-approved protocols following standard guidelines described at www.zfin.org and in the Guide for the Care and Use of Laboratory Animals, 8th Edition.

Bacterial strains

The bacterial strains used in this study are listed in Table 3.1. Bacteria used for infecting zebrafish and mice were grown statically at 37°C for 24 h in 12 ml modified M9 minimal medium (6 g/l Na_2HPO_4, 3 g/l KH_2PO_4, 1 g/l NH_4Cl, 0.5 g/l NaCL, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 0.1% glucose, 0.0025% nicotinic acid, 0.2% casein amino acids, and 16.5 µl thiamine in H_2O, pH 7.2). F11 carrying pGEN-GFP(LVA) was grown in medium containing ampicillin (50 µg/ml). Targeted deletion of *fliC* in F11 and CFT073 were generated using lambda Red-based recombination as described previously [47]. Expression constructs pBF14 and pBF15 were made using the low-copy-number plasmid pGEN-MCS and standard molecular biology techniques as in [18]. Primers for the generation and confirmation of the strains and plasmids used in this chapter can be found in Table 3.2.

Zebrafish embryos

Wild type AB zebrafish were maintained as breeding colonies on a 14-h/10-h light/dark cycle. Embryos were collected as mixed egg clutches and raised at 28.5°C in E3 medium (5 mM NaCl, 0.27 mM KCl, 0.4 mM $CaCl_2$, 0.16 mM $MgSO_4$; pH 7.4) containing 0.000016% methylene blue as an antifungal agent.

Infection of zebrafish embryos

48 hpf embryos were manually dechorianated, briefly anesthetized with 0.77 mM ethyl 3-aminobenzoate methanesulfonate salt (tricaine) (Sigma-Aldrich), and embedded in 0.8% low melt agarose (MO BIO Laboratories) without tricaine. After the agarose solidified, embryos were immersed in E3 media lacking methylene blue. Prior to injection, 1 ml of bacterial culture was pelleted, washed with 1 ml sterile PBS, and resuspended in PBS to obtain ~$1X10^9$ CFU/ml PBS. One nl of this bacterial suspension was microinjected into the bloodstream via the circulation valley using an Olympus SZ61 or SZX10 stereomicroscope together with a YOU-1 micromanipulator (Narishige), a Narishige IM-200 microinjector, and a JUN-AIR model 3-compressor. For each experiment, the average CFU per injection was determined by adding 10 1-nl drops to 1 ml of 0.7% NaCl, which was then serially diluted and plated on Luria-Bertani (LB) agar plates. Mock-infected controls were inoculated with 1 nl sterile PBS. Following injection, embryos were removed from agar and placed individually into wells of a 48-well plate (Nunc) containing E3 medium and incubated at 28.5°C. For lethality assays, death was defined by absence of heart contraction and blood flow.

Enumeration of bacterial numbers in zebrafish embryos

Embryos were homogenized at the indicated time points in 500 µl of PBS containing 0.5% Triton X-100 using a mechanical PRO 250 homogenizer (PRO Scientific). Homogenates were serially diluted and plated on LB agar plates, which were then incubated overnight at 37°C.

RNA isolation from zebrafish embryos

Pools of 15-20 embryos were manually homogenized in 1 ml QIAzol Lysis Reagent (Qiagen) and total RNA extracted using the Qiagen RNeasy Plus Universal Kit according to the manufacturer's instructions. Genomic DNA was removed by the gDNA Eliminator Solution (Qiagen).

cDNA synthesis and qRT-PCR

cDNA was synthesized from 2 µg of RNA using the SuperScript III First-Strand Synthesis System (Invitrogen) in a 20 µl reaction volume. Following assembly of the master mix according to manufacturer instructions, samples were incubated at 25°C for 5 min, 55°C for 45 min, and 70°C for 15 min. Complimentary RNA was then removed by addition of RNase H (Invitrogen) for 15 min at 37°C.

qRT-PCR was performed on a LightCyler 480 instrument (Roche) following manufacturer recommendations. Cycling parameters were 95°C for 8 min to activate the polymerase followed by 40 cycles of 95°C for 4 sec, 60°C for 6 sec, and 72°C for 6 sec. Fluorescence measurements were taken at the end of

each cycle. Melt curve analysis was performed to ensure that no primer dimers were amplified. All reactions were performed in technical duplicate. Sequences for the forward and reverse primers are listed in Table 3.2. Results were normalized to transcript levels of the housekeeping gene *ef1*α (elongation factor 1-alpha) using $\Delta\Delta C_t$ analysis.

Semipurified bacterial flagellin preparation

Overnight bacterial cultures were back diluted 1:100 into 20 ml of tryptone broth and grown shaking (180 rpm) at 30°C to midlog. Motility was verified by microscopy prior to proceeding. Cultures were spun down at 6000g for 10 min at 4°C and resuspended in 3 mL sterile PBS. Suspensions were then passed between two syringes connected with 0.28mm I.D. polyethylene tubing (Becton Dickson) to shear flagella. Following shearing, samples were spun down at 6000g for 10 min at 4°C and supernatant containing flagella collected. Equal loading of flagellar protein for TLR reporter assays was verified by immunoblotting for fliC (Abcam anti-Flagellin; 1:10,000) following standard Western blotting protocols.

Quantification of flagella expression in infected embryos

Embryos were infected with approximately 2000-2500 CFU CFT073/p*fliC*-lux and F11/*fliC*-lux as described above. Following infection, embryos were individually placed into wells of a white-walled 96 well plate containing E3 medium and incubated at 28.5°C. Luminescence was measured at each time

point using a BioTek Synergy H1 plate reader.

Quantification of TLR5 activation

TLR5 stimulation was assayed using HEK-Blue mTLR5 cells (Invivogen), a SEAP reporter line expressing mouse TLR5. Briefly, cells were suspended at a concentration of 140,000 cells/mL in HEK-Blue detection media and 180 µl (25,000 cells/well) placed into wells of a 96 well plate. Cells were then stimulated with 20 µl semipurified flagella or PBS as a control. After 15 hours, absorbance (640 nm) was measured using a BioTek Synergy H1 reader. For neutralizing antibody experiments, 1 µg/ml anti-mTLR5 or control IgG2a antibody were added to cells and allowed to incubate for 1 hour prior to the addition of flagella.

TLR5a/b morpholino experiments

Morpholino oligonucleotides (Gene Tools) were used to block translation of *tlr5a* and *tlr5b*. Using previously published oligonucleotide sequences [42, 43] 0.85 ng (0.1 mM) *tlr5a* morpholino and 4.2 ng (0.5 mM) *tlr5b* morpholino were injected into one-cell stage zebrafish embryos. To control for nonspecific morpholino effects, 0.6 mM of a standard control morpholino was injected into one-cell stage embryos. Sequences for morpholino oligonucleotides can be found in Table 3.2.

Generation of vectors for recombinant protein purificiation

pBF12, pBF16, pAB1, and pAB2 were generated by subcloning the coding sequences of CFT073 FliC, CFT073 FliC176-497, F11 FliC, and F11 FliC176-

466 sans stop codon, respectively, into parent vector pSF-CMV-NH2-InsulinSP1-NcoI (Oxford Genetics). A 6xHis tag was added to the C terminal end of each coding sequence for recombinant protein purification. The parent vector uses the mammalian CMV promoter to drive strong gene expression and adds a secretory signal peptide to the N-terminus. This signal causes the translocation of the nascent protein to the endoplasmic reticulum, where the signal peptide is cleaved off and the peptide is secreted into the medium by bulk flow exocytosis. pBF12(V488I) and pBF16(I528V) were generated using the QuikChange II Site-Directed Mutagenesis Kit (Agilent) following the manufacturer instructions. Primer sequences for the generation these constructs are provided in Table 3.2.

Recombinant protein generation by 293T cells

293T cells were maintained in DMEM containing 10% heat-inactivated FBS, 2 mM L-glutamine and 1% Penicillin/Streptomycin in a humidified incubator with 5% CO2. For transfection, 293T cells were seeded at 30% confluency with the goal of being 60% confluent at the time of transfection. pBF12, pBF16, and the control vector pSF-CMV-NH2-InsulinSP1-Nco1 were transiently transfected using calcium phosphate to mediate transfection and media containing recombinant protein was collected at 72 hours post-transfection. Levels of recombinant protein were determined by SDS-PAGE and immunoblot against Flagellin (Abcam; 1:10,000) as in [48].

Statistical analysis

Except where indicated, *p* values were calculated by two-tailed Student's t-tests, Mann-Whitney U tests, or log-rank tests using Prism 6.0e software (GraphPad Software). Values of less than 0.05 were defined as significant for all experiments.

Acknowledgements

I am grateful to Harry Mobley (University of Michigan, Ann Arbor, MI) for p*fliC*-lux, June Round (University of Utah, Salt Lake City, UT) for sharing reagents, David Blaire (University of Utah, Salt Lake City, UT) for advice on the isolation of flagella, and Jonathan Kagan (Harvard Medical School, Boston, MA) for insightful conversations regarding the purification of recombinant bacterial protein from mammalian cells. I would also like to thank Brittany Fleming for her assistance in generating and verifying the *flic* mutants and the creation of pBF12 and pBF16.

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Table 3.1. Bacterial strains and plasmids used in Chapter 3.

***E. coli* Strain**	**Description**	**Source or Reference**
F11	Cystitis isolate (O6:K2:H31)	[49]
CFT073	Urosepsis isolate (O6:K2:H1)	[50]
pfliC-lux	Encodes *fliC* driven luciferase reporter, Amp^R	[18]
pKM208	Encodes IPTG inducible lambda Red recombinase, Amp^R	[47]
CFT073Δ*fliC*	CFT073 *fliC::kan*	This study
F11Δ*fliC*	F11 *fliC::kan*	This study
pBF14	Encodes FliC from F11	This study
pBF15	Encodes FliC from CFT073	This study
pBF12	Encodes CMV driven F11_FliC with a N-terminal insulin secretion sequence and C-terminal 6xHis tag, Kan^R	This study
pBF16	Encodes CMV driven CFT073_FliC with a N-terminal insulin secretion sequence and C-terminal 6xHis tag, Kan^R	This study
pAB1	Encodes CMV driven CFT073_FliC(176-497) with a N-terminal insulin secretion sequence and C-terminal 6xHis tag, Kan^R	This study
pAB2	Encodes CMV driven F11_FliC(176-466) with a N-terminal insulin secretion sequence and C-terminal 6xHis tag, Kan^R	This study
pBF12(V488I)	Amino acid substitution of isoleucine for valine at reside 488 in pBF12	This study
pBF16(I528V)	Amino acid substitute of valine for isoleucine at reside 528 in pBF16	This study

Table 3.2. Oligonucleotides used in Chapter 3.

Primer	Sequence (5'-3')
CFT073*Δflic* Knock Out	
Forward	CGTAATCAACGACTTGCAATATAGGATAACGAATCTGTGTAGGCTGGAGCTGCTTCG
Reverse	GGCGTTGCCGTCAGTCTCAGTTAATCAGGTTACGGCGACATATGAATATCCTCCTTAG
CFT073*Δflic* Confirmation	
Forward	CGACAGACGATAACAGGGTTG
Reverse	ATCCGGCCTACAAAAATGTG
F11*Δflic* Knock Out	
Forward	CGTAATCAACGACTTGCAATATAGGATAACGAATCTGTGTAGGCTGGAGCTGCTTCG
Reverse	GGCGTTGCCGTCAGTCTCAGTTAATCAGGTTACAACGACATATGAATATCCTCCTTAG
F11*Δflic* Confirmation	
Forward	CGACAGACGATAACAGGGTTG
Reverse	CTTACCCGGCCTACAAAATG
zEF1α	
Forward	TGAGCGTGGTATCACCATTG
Reverse	CAACACCACCAGCAACAATC
zIL1β	
Forward	TGGACTTCGCAGCACAAAATG
Reverse	CACTTCACGCTCTTGGATGA
zIL8	
Forward	TGTGTTATTGTTTTCCTGGCATTTC
Reverse	GCGACAGCGTGGATCTACAG
zTLR5a Morpholino	AAAGTGTATGTAGCTGCCATTCTGG
zTLR5b Morpholino	TGAATGTATATCCCATTCTGTGAGC
Control Morpholino	CCTCCTACCTCAGTTACAATTTATA
pBF12	
Forward	GGCTGAGGTCGCCATGGGTATGGCACAAGTCATTAATAC
Reverse	GATAACTCGAGTTAGTGGTGGTGGTGGTGGTGTCCACCCTGCAGCAGAGACAGAA
pBF16	
Forward	GGCTGAGGTCGCCATGGGTATGCTGAATGGTTTTAACGTGAA
Reverse	ATAACTCGAGTTAGTGGTGGTGGTGGTGGTGTCC ATCTTTTGTTACTGTGCCTTC
pBF14/pBF15	GATCGAAGCTTGTTATCGGCCTGAATTGCGC GATTGCCATTGGTTAACCCTGCAGCAGAGACAG
pAB1	
Forward	GGCTGAGGTCGCCATGGGTATGCTGAATGGTTTTAACGTGAA
Reverse	GATAACTCGAGTTAGTGGTGGTGGTGGTGGTGTCCATCTTTTGTTACTGTGCCTTC
pAB2	
Forward	GGCTGAGGTCGCCATGGGTATGCTGAGTGGGTTTAATGTGAA
Reverse	GATAACTCGAGTTAGTGGTGGTGGTGGTGGTGTCCATCGGCGGTGGTTGCAGCTT
pBF12(V488I)	
Forward	TTTCGCTCCTCCTTGGGTGCGATACAAAACCGTCTGGATTCTGCGG
Reverse	CCGCAGAATCCAGACGGTTTTGTATCGCACCCAAGGAGGAGCGAAA
pBF16(I528V)	
Forward	ATTCCGTTCATCCCTGGGGGCTGTCCAAAACCGTTTGGA
Reverse	TCCAAACGGTTTTGGACAGCCCCCAGGGATGAACGGAAT

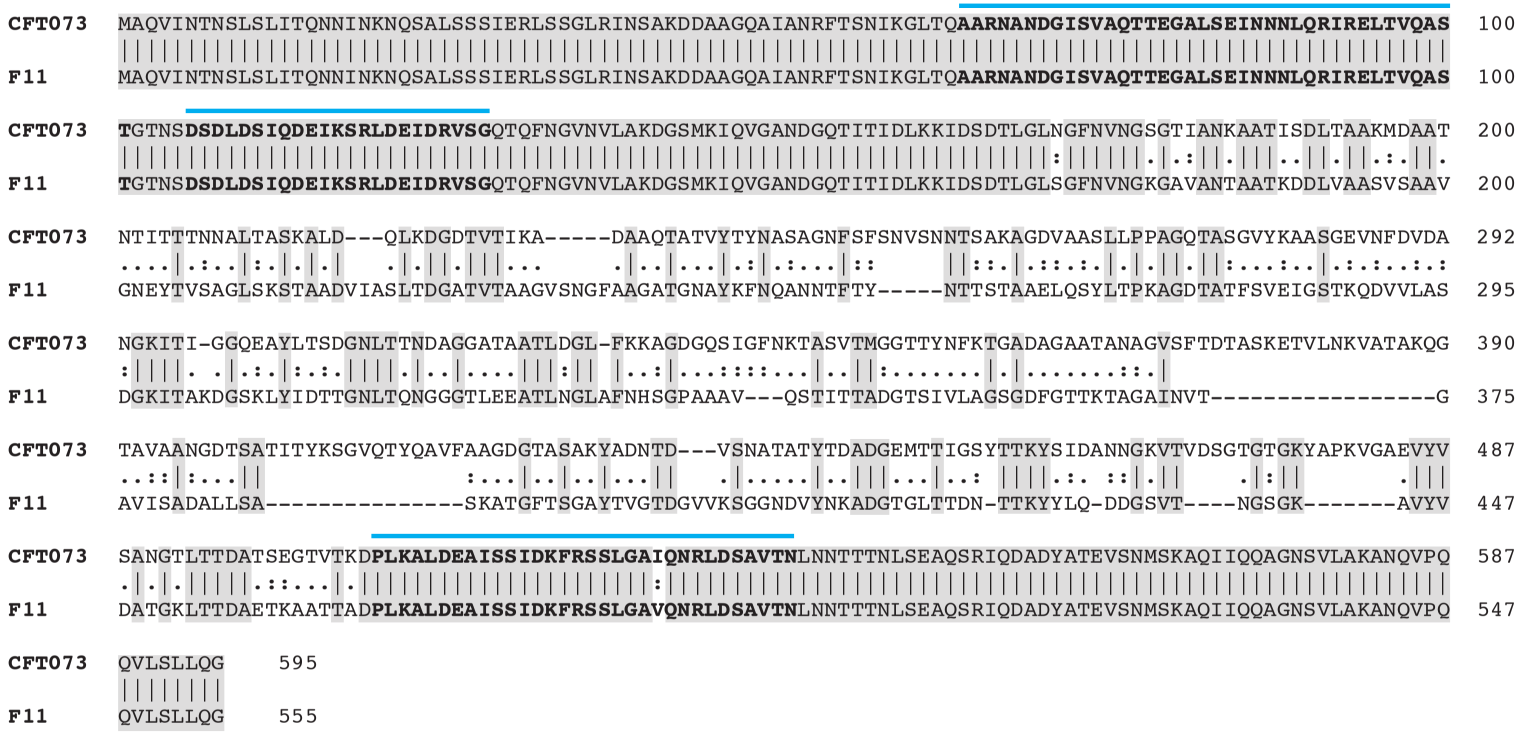

Figure 3.1. Protein sequence alignment of FliC from CFT073 (H1) and F11 (H31).
Sequences share 67.3% similarity. Identical residues are marked in gray. Predicted TLR5 binding regions are indicated with blue lines and bold text.

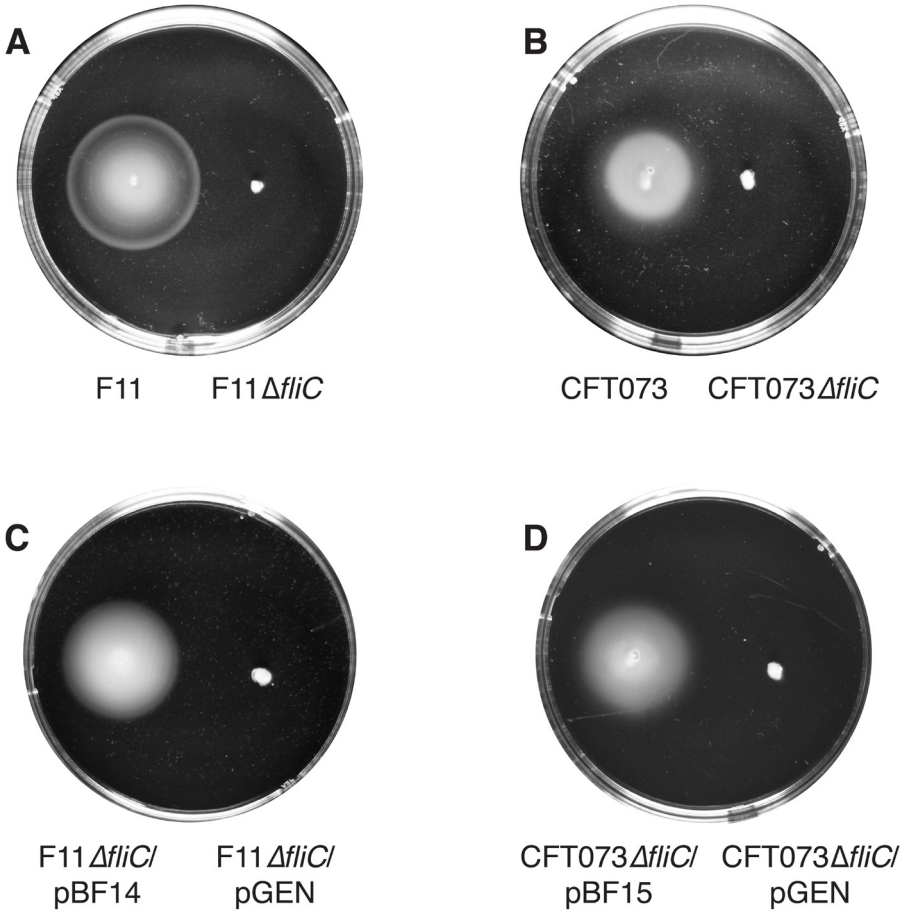

Figure 3.2. Functional verification and complementation of *fliC* mutants. (**A-B**) Deletion of *flic* in F11(**A**) and CFT073 (**B**) renders both pathogens immobile on 0.1% LB agar plates. Wild type strains are also pictured as controls. (**C**) Complementation of F11Δ*fliC* with pBF14 restores motility on 0.1% LB agar plates, while the mutant carrying the pGEN empty vector remains immobile. (**D**) Complementation of CFT073Δ*fliC* with pBF15 restores motility on 0.1% LB agar plates, while the mutant carrying the pGEN empty vector remains immobile.

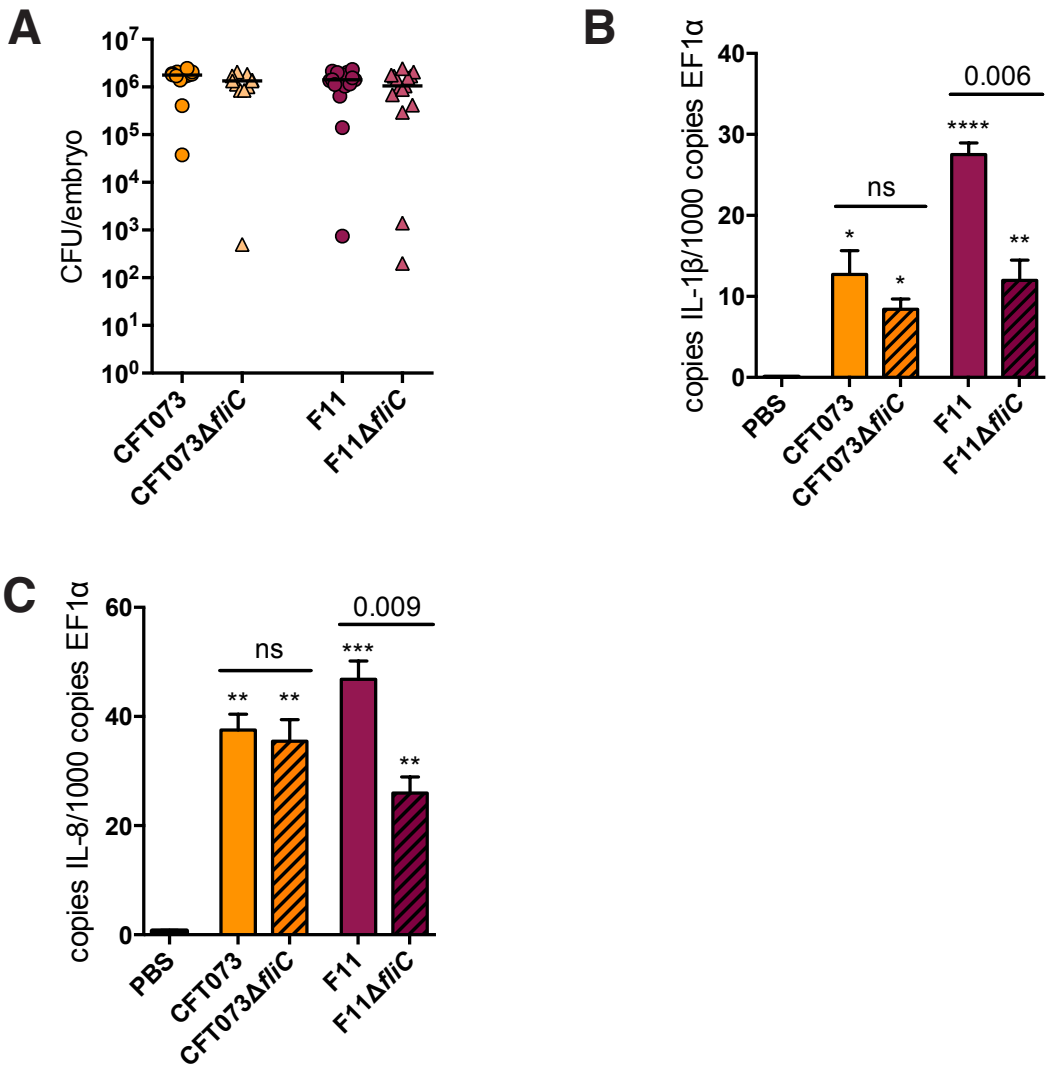

Figure 3.3. Flagellin contributes to the inflammatory response of F11, but not CFT073 during sepsis.

(**A**) Bacterial burden in infected embryos at 12 hpi with ~1,000 CFU. Data were pooled from two independent experiments. Bars denote median values; n = 10 embryos. No significant differences were detected between wild type and *fliC* mutant strains, as determined by Mann-Whitney U tests.

(**B-C**) qRT-PCR analysis of the specified cytokine transcripts at 12 hpi with PBS or ~1,000 CFU of the indicated E. coli strains. Bars indicate mean results ± SEM from 3 pools of ≥ 18-20 embryos. qRT-PCR for each pool was carried out using technical duplicates. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001 versus PBS-injected controls, as determined by Student's t tests. P values for wild type vs. *fliC* mutant samples are also indicated.

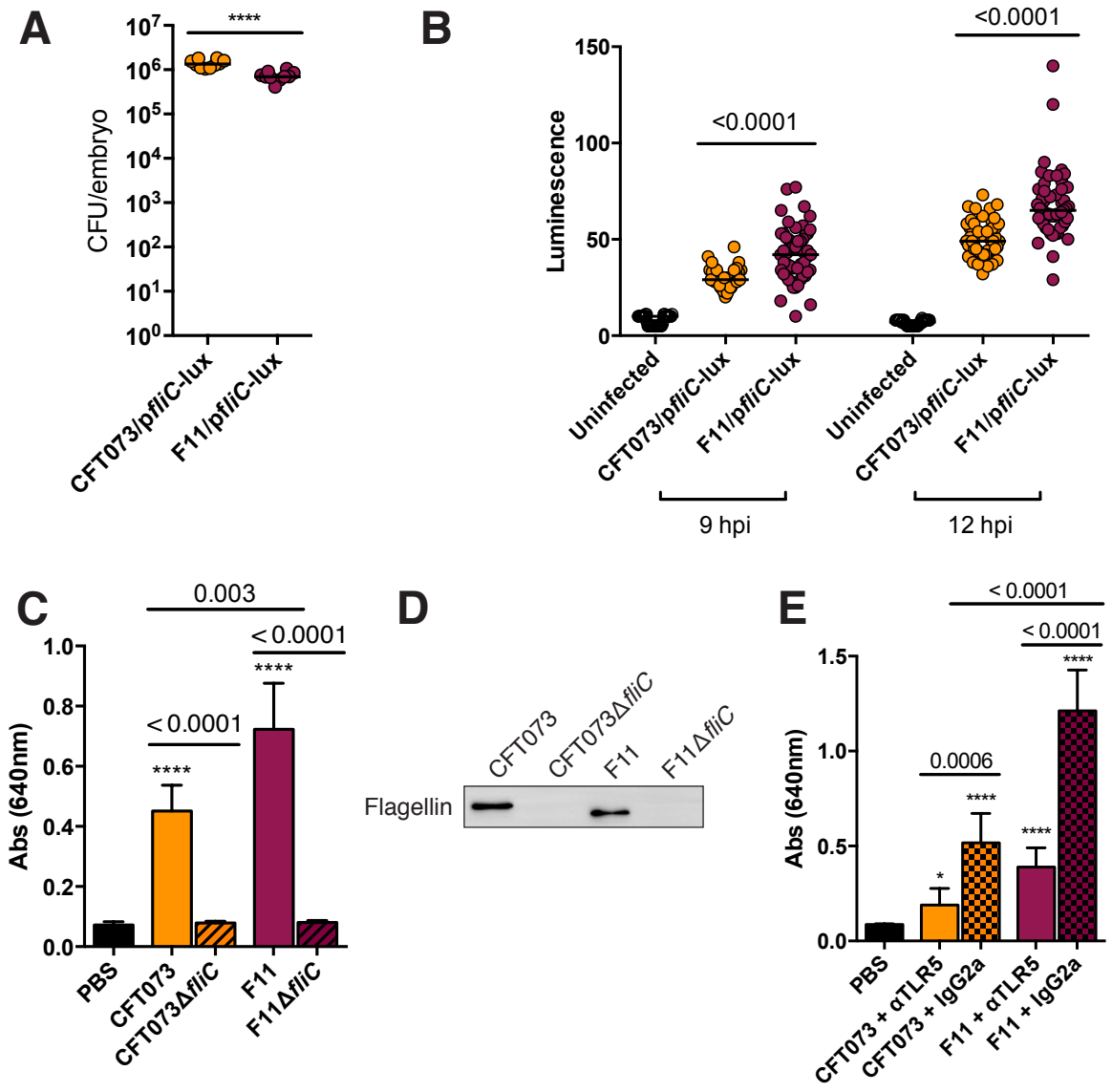

Figure 3.4. Differences in inflammation between CFT073 and F11 result from variable flagellin expression and differential stimulation of TLR5.

(**A**) Bacterial burden in infected embryos at 12 hpi following innoculation with ~5000 CFU. Data were pooled from two independent experiments. Lines mark median values; n = 10 embryos. ****$P \leq 0.0001$, as calculated by Mann-Whitney U test. (**B**) *fliC* expression in infected embryos at 9 and 12 hpi. Lines mark median values; n = 30-50 embryos pooled from two independent experiments. *P* values calculated by Mann-Whitney U tests. (**C**) Immunoblot of semipurified flagella from CFT073, CFT073Δ*fliC*, F11, and F11Δ*fliC*.

(**D-E**) TLR5 stimulation by semipurified flagella, as measured using HEK-Blue mTLR5 reporter cells. Bars represent mean results ± 95% CI from two independent experiments with three replicates. ****$P \leq 0.0001$ versus PBS control, as determined by Student's t tests.

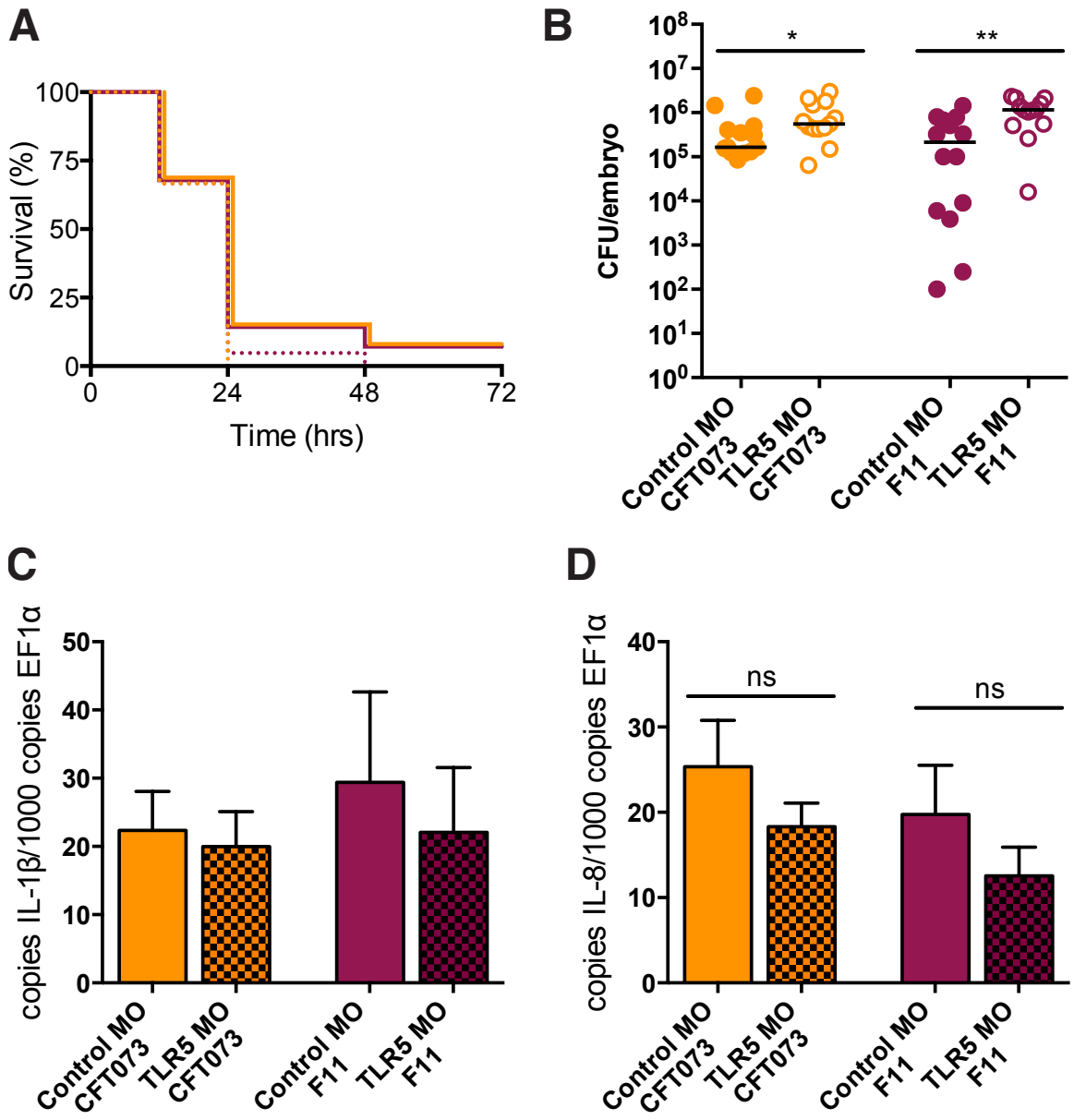

Figure 3.5. Knockdown of TLR5 increases bacterial burden during sepsis, but does not affect cytokine levels.

(**A**) Representative Kaplan-Meier survival curve of control or TLR5 morphant zebrafish embryos infected with CFT073 (solid and dotted orange lines) or F11 (solid and dotted maroon lines); n=12-18 embryos. (**B**) Bacterial burden in infected embryos at 12 hpi with ~1,000 CFU. Data were pooled from three independent experiments. Bars denote median values; n = 14 embryos. **P* ≤ 0.05, ***P* ≤ 0.01 as determined by Mann-Whitney U test. (**C-D**) qRT-PCR analysis of IL-1β (**B**) and IL-8 (**C**) at 12 hpi with ~1,000 CFU of the indicated E. coli strains. Bars indicate mean results ± SEM from 4 pools of ≥ 18-20 embryos. qRT-PCR for each pool was carried out using technical duplicates. No significant differences were detected between TLR5 morphants and control morphants for either CFT073 or F11 using Student's t tests.

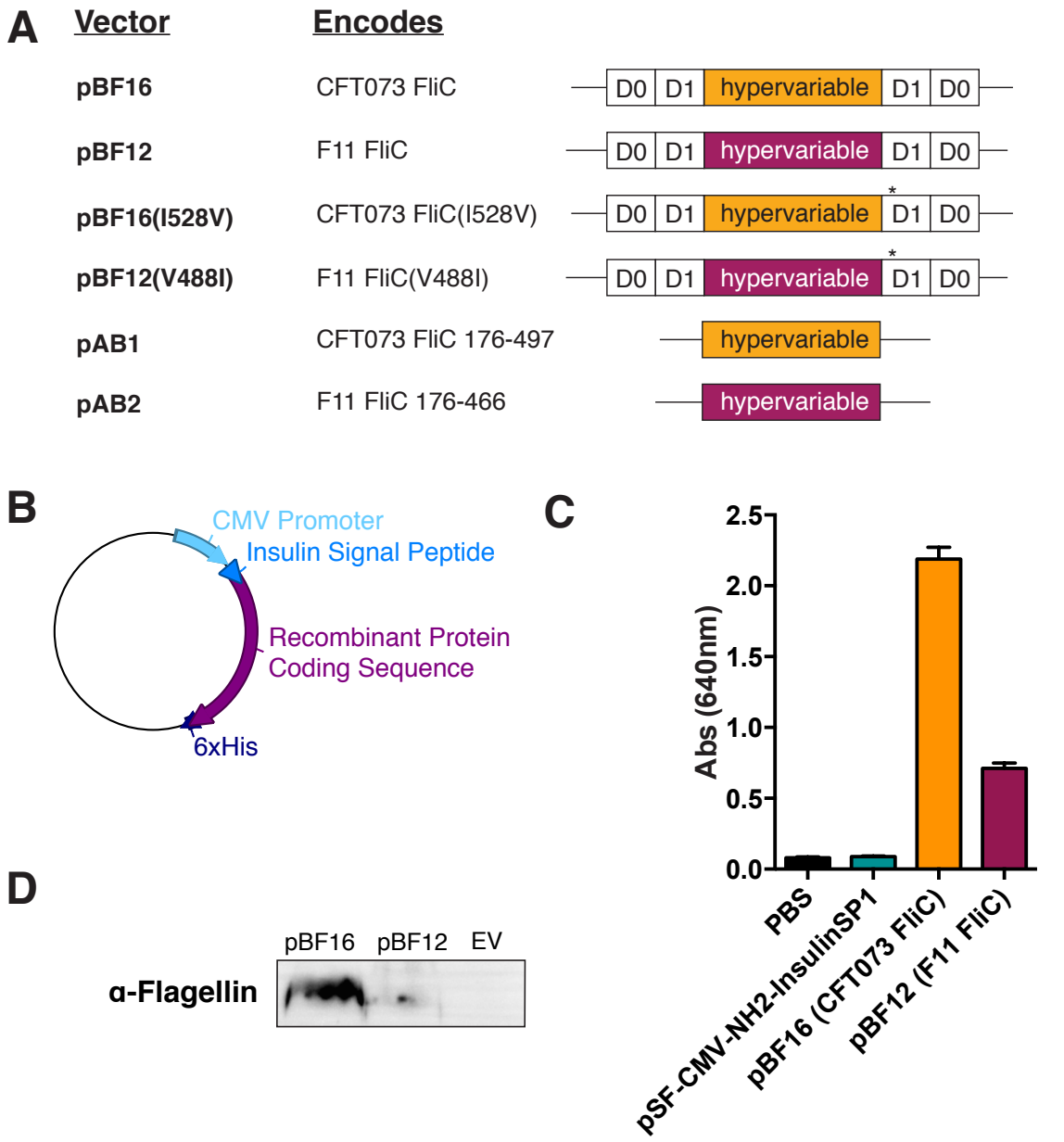

Figure 3.6. Recombinant flagellin production by 293T cells.
(**A**) Illustration of constructs created to determine the molecular basis for differential stimulation of TLR5 by FliC variants from CFT073 and F11.
(**B**). Each construct from (**A**) was cloned into parent vector pSF-CMV-InsulinSP1-Nco1 which features CMV-driven expression of the recombinant coding sequence and a 3' his tag. Recombinant protein is secreted into the medium of transfected cells as a result of the 5' insulin signal peptide.
(**C**) TLR5 stimulation by recombinant flagellin, as measured using HEK-Blue mTLR5 reporter cells. Bars represent mean results ± SD from three replicates.
(**D**) Immunoblot of media from 293T cells transfected with pBF12, pBF16, or EV (pSF-CMV-NH2-InsulinSp1).

CHAPTER 4

GENERATION OF NOVEL ZEBRAFISH LINES FOR THE STUDY OF INTERLEUKIN 1β AND INTERLEUKIN 10

Abstract

The immune response to pathogens or tissue damage involves the rapid activation of immune cells and generation of pro-inflammatory cytokines (e.g., IL-β, IL-6, TNFα). However, excess inflammation can be destructive and detrimental to the host so the immune system has evolved parallel anti-inflammatory molecules (e.g., IL-10) and pathways to regulate and curb production of inflammatory molecules and prevent damage to the host. The zebrafish is an attractive, emerging model for the study of host-microbe interactions and inflammation (see Chapter 1), but is currently hampered by the limited availability of tools in these disciplines such as knock out lines in immune-associated genes and transgenic reporter lines for immune cell types and mediators. Within this chapter I will discuss the generation of an IL-10 deficient zebrafish and transgenic lines placing IL-1β and IL-10 under inducible control to better understand the roles of these molecules during infection and other physiological processes.

Introduction

Interleukin-10: the prototypical anti-inflammatory cytokine

IL-10 is part of a greater family of cytokines (including IL-10, IL-19, IL-20, IL-22, IL-24, and IL-26) based upon similarities in their genomic architecture, protein structures, and the receptor complexes used [1]. IL-10 itself is a small 20 kDa protein whose secondary structure forms a compact, bundle-like shape comprised of 6 α-helices creating a hydrophobic core stabilized by 2 disulfide

bridges essential for maintaining the structure and biological activity of the cytokine. Functioning as a homodimer, the main *in vivo* source of IL-10 is monocytes, macrophages, and T-cell subsets. However it is also synthesized by other immune cells such as dendritic cells, B cells, natural killer cells, neutrophils, and other granulocytic immune cells [2]. The receptor for IL-10 is comprised of two IL-10R1 and two IL-10R2 molecules and is expressed predominantly on macrophages and monocytes, making them the main cellular targets for IL-10. IL-10-receptor binding induces JAK/STAT signaling, including the downstream activation of Suppressor of cytokine signaling 3 (SOCS3) to mediate its downstream effects.

IL-10 is one of the most important anti-inflammatory and immunosuppressive cytokines and is produced by immune cells following their activation. It functions largely to suppress the key functions of macrophages, including downregulating the release of pro-inflammatory mediators IL-1β and TNFα, inhibiting antigen presentation, and altering phagocytosis and microbial killing [2]. Additionally, it inhibits the expression of MHC class II and associated co-stimulatory molecules and inhibits the synthesis of Prostaglandin-endoperoxide synthase 2.

IL-10 is implicated in the pathogenesis of myriad infections and inflammatory conditions. The absence of IL-10 in mice either by disruption of the *il10* gene or antibody blockade of the receptor leads to improved clearance of many bacterial, fungal, and viral pathogens [reviewed in 2, 3]. Occasionally this is

not accompanied by an increase in immunopathology [4, 5]. However more often the absence of IL-10 leads to severe immunopathology [6-10]. Given that the absence of IL-10 improves pathogen clearance, it is not surprising that many infectious microbes such as *Mycobacterium tuberculosis* and *Trypanosoma cruzi* have evolved mechanisms to selectively upregulate IL-10 during infection [8, 10]. Some viruses (e.g., *Herpesviridae* and *Poxviridae*) take a more direct approach and encode IL-10 homologs within their genomes to create a more favorable niche for themselves [11].

The role of IL-10 during sepsis is complex given its pleotropic immunosuppressive properties. On one hand, IL-10 deficient mice show enhanced mortality and increased organ damage in an *E. coli* model of sepsis on account of increased levels of inflammatory cytokines such as TNFα [7]. However, exogenous administration of IL-10 during sepsis has produced heterogeneous results [discussed in 12] and there are additional concerns regarding IL-10's suppression of T cell responses during recovery from sepsis.

Interestingly, IL-10 secreted by monocytes keeps neural stem cells in an undifferentiated state, blocking neurogenesis and partially explaining why regenerative neurogenesis does not take place following injury of the adult brain [13, 14]. IL-10 plays a key modulatory role in tolerizing the immune system to resident enteric bacteria as IL-10 deficient mice spontaneously develop inflammatory colitis [15], a phenomenon that does not happen in mice maintained under germ free conditions [16]. IL-10 deficiency and the associated rise in

inflammation have also been linked with the development and pathogenesis of psoriasis, rheumatoid arthritis, inflammatory bowel disease, and systemic lupus erythematosus [17, 18]. While recombinant IL-10 has been investigated as therapy for many of these conditions, the results have been heterogeneous and overall, not particularly effective during clinical trials [19].

Zebrafish IL-10 (zIL-10) is highly similar to mammalian IL-10, sharing the same genomic architecture of five exons and four introns and secondary structure comprised of six α-helices [20]. The four cysteine residues essential for biological function in mammalian IL-10 are also conserved in zIL-10. Despite these similarities, there are currently no transgenic lines or characterized mutants for the study of this key anti-inflammatory mediator in zebrafish. Within this chapter I will describe the generation of an IL-10 deficient zebrafish as well as a transgenic line placing IL-10 under inducible control.

Interleukin-1β: a key inflammatory mediator

IL-1β is a master inflammatory cytokine of local and systemic inflammation. As such, the levels of IL-1β are tightly regulated. IL-1β mRNA is not constitutively expressed and most of the IL-1β transcripts produced are degraded and never translated. Blood monocytes, tissue macrophages, and dendritic cells are the primary sources of IL-1β, but it can also produced by B cells, NK cells, and platelets. IL-1β is unique among cytokines in that it is produced as a pro-peptide that requires processing by Caspase-1 for activity. Caspase-1 itself requires activation by the inflammasome, a multiprotein complex, to initiate the

inflammatory cascade. While activation of IL-1β occurs predominantly via Caspase-1, Proteinase-3 or Elastase can also activate IL-1β [21]. Following IL-1β binding to its cognate receptor, Interleukin-1 receptor (IL1R), the cytoplasmic domain of IL1R recruits adapter protein MyD88 and downstream inflammatory programs are initiated by NFκB and AP-1. Nearly all microbial products induce the production of IL-1β via TLR signaling, as do activated complement components and other cytokines (i.e., TNFα). IL-1β can also induce itself, forming a positive inflammatory feedback loop and leading to autoinflammation [22]. The systemic symptoms of IL-1β expression include fever, inflammatory pain hypersensitivity, vasodilation, and hypotension. Patients treated with a low dose of IL-1β also exhibit a loss in appetite, muscle and joint pain, fatigue, as well as sleep and gastrointestinal disturbances [23].

While IL-1β deficient mice exhibit no overt phenotypes during development, homeostasis, or resistance to endogenous microbial flora, IL-1β has myriad indirect effects on immune cells [24, 25]. The presence of IL-1β increases the expression of cellular adhesion molecules, thus promoting the infiltration of inflammatory cells into tissue from the circulation. Exposure to IL-1β induces the differentiation of bone marrow stem cells into myeloid progenitors, prolongs the lifespan and effector function of neutrophils, and increases the number of circulating neutrophils. It is also essential for the generation of Th17 responses and plays a role in antibody production. In models of local or systemic inflammation, IL-1β deficiency typically results in increased susceptibility to

infections, but reduced disease severity [24]. Importantly, infants born with a loss of function in IL-1 receptor agonist (IL-1RA), a natural inhibitor of IL-1β, die early in life due to overwhelming inflammation of the skin and joints with large amounts of infiltrating neutrophils and high IL-17 levels.

IL-1β plays a significant role in the pathogenesis of many inflammatory disorders including rheumatoid arthritis, gout, systemic juvenile idiopathic disease, type ii diabetes, and Behcet's disease. IL-1β is also a key participant in familial autoinflammatory diseases such as cryopyrin-associated periodic syndrome and familial Mediterranean fever that are marked by increased inflammation mediated by cytokines of the innate immune system, without the presence of autoantibodies or self-reactive T cells. Surprisingly, a crucial role for IL-1β during cancer metastasis has been established. In IL-1β deficient mice, vascular endothelial growth factor cannot stimulate the formation of blood vessels, so malignant melanoma cells do not spread and mice rarely develop tumors. The tumors that do form develop significantly slower and are marked by their absence of leukocyte infiltration [26].

Considering the significant contributions that IL-1β makes to a multitude of diseases, it is not surprising that it is an emerging therapeutic target. There are three currently approved therapeutics for reducing IL-1β activity and numerous others in clinical trials [discussed in 27]. In addition to being a valuable therapeutic for the chronic inflammatory diseases mentioned above, targeting IL1 has also proved beneficial in cases of acute ischemic inflammation following

myocardial infarction, stroke, and acute lung injury. Blocking IL1 also has utility in the treatment of chronic inflammatory conditions that are refractory to glucocorticoids and TNF blockers. IL-1 blockade was investigated as a sepsis therapeutic given the sharp increase in IL-1β levels during sepsis [28-31]. Though initially promising, reducing IL-1β did not significantly affect patient mortality, which was thought to be due to the heterogeneity in patients with sepsis.

Zebrafish IL-1β (zIL-1β) is conserved compared to its mammalian ortholog, including structural conservation of the critical βtrefoil fold and it is transcriptionally upregulated during infection and following inflammatory stimuli [Figure 2.11, 32, 33-35]. Additionally, zebrafish possess two Caspase 1 orthologs (Caspase A and Caspase B) that are both capable of processing zIL-1β [36]. In this chapter I will discuss the creation of a novel zebrafish line placing IL-1β under the control of *hsp70* and an unsuccessful attempt to create an IL-1β deficient zebrafish.

Results

Use of TALENs to induce somatic mutations in zIL-10

Reverse genetics to elucidate the function of specific genes in zebrafish has been problematic up until recent years. One previous approach was to attenuate gene function in embryos using antisense morpholino oligonucleotides. However, morpholinos could only be used to transiently reduce gene expression and were limited to embryos. Additionally, morpholinos frequently had detrimental

off-target effects [37] and, more alarmingly, often failed to phenocopy genomic mutants for the same gene target [38]. Another approach for perturbing selected gene function was the use of zinc finger nucleases to specifically induce somatic mutations in the zebrafish by targeting a nuclease to a desired location. While initially promising, the rules of zinc finger protein binding to DNA are incompletely understood and zinc finger nucleases did not have the ability to target any desired genomic locus [39, 40].

As a solution, Transcription Activator-Like Effector (TALE) proteins have been developed to specifically target genomic regions of interest. TALEs were originally identified as injected effectors of the pathogenic plant bacteria *Xanthomonas.* These effectors bind to the promoters of anti-inflammatory genes with high affinity and specificity, causing upregulation of these transcripts and allowing the bacteria to initiate a productive infection [41]. DNA binding of TALE molecules is mediated by a collection of 33-34 amino acid DNA recognition binding motifs, where an individual motif recognizes and binds a single nucleotide [42, 43]. TALENs, which are the fusion of TALE proteins with the sequence independent nuclease FokI, function as obligate heterodimer endonucleases where a “left” and “right” TALE protein each recognizes a unique DNA site separated by a spacer region [44, 45]. Targeted DNA cleavage by FokI is only achieved when both molecules are bound with the correct spacing and orientation and are effective in inducing genomic mutations in zebrafish and other systems [46, 47]. TALENs induce DNA double strand breaks in the target site

that are repaired by host machinery in a process called nonhomologous end joining. This process is error prone and often produces small (2-20 base pair) genomic insertions or deletions (indels) that, when occuring in coding regions, disrupt the coding sequence and can cause premature stops, leading to null mutations.

The *il10* locus of zebrafish is on chromosome 12 and consists of 5 coding exons. To generate targeted mutations of *il10*, we used TALENs directed against the start of exon 3 (Figure 4.1A). This site was chosen with the guidance of the University of Utah Mutation and Detection Core who also designed and synthesized the plasmids encoding the left and right TALEN monomers. To induce mutation in *il10*, we synthesized and injected equal amounts of mRNA encoding each TALEN monomer into one-cell zebrafish embryos (Figure 4.1B). TALEN activity and the presence of mutations were confirmed in our G_0 population using high resolution melt analysis (HRMA) on a portion of the injected embryos at 24 hpf. HRMA consists of amplifying small (50-75) bp DNA fragments in the presence of a saturating dye such as LCGreen that binds to DNA, but is fluorescent only when bound to double-stranded DNA. After the amplification, the sample is heated until the two strands fall apart, quenching fluorescence. Any changes in DNA sequence result in detectable differences in the melt curve.

We found that 90% of TALEN-injected embryos showed evidence of mutation by HRMA (Figure 4.2A). When our G_0 population reached sexual

maturity, we outcrossed them to wild type fish to determine if mutations reached the germ line and found that 81% of our G_0 adults showed evidence of germ line mutation transmission. In line with published observations, most G_0 founders were capable of transmitting multiple mutant alleles. On average, 31% of G_1 progeny had mutation in *il10* (range 0-75%).

To identify null mutants, we prescreened for $il10^{+/\Delta}$ G_1 adults with HRMA as above and then PCR amplified and Sanger sequenced the TALEN-targeted region of *il10*. Since the individual fish being genotyped were heterozygous for a mutant allele, the resulting sequence file starts with a series of single peaks, but then turns to multiple peaks at the site of the indel (Fig 4.2B). Using the web-based Poly Peak Parser software developed by the Yost lab at the University of Utah (http://yosttools.genetics.utah.edu/PolyPeakParser/) which separates wild type and mutant sequences from each other, we identified G_1 individuals with 5 bp ($il10^{\Delta 5}$) and 13 bp ($il10^{\Delta 13}$) deletions in the start of exon 3 of *il10* (Fig 4.2C).

Use of unlabeled probe melting analysis for genotyping of *il10* mutant zebrafish

While the $il10^{\Delta/\Delta}$ fish were viable, we decided to maintain our breeding adults as heterozygotes to avoid potential complications that the *Il10* knockout mouse is known to suffer from such as spontaneous colitis [48]. This strategy has the additional benefit of providing wild type siblings as controls. However, we sought to develop a more robust genotyping protocol because most of our experiments would be on 2 dpf embryos and thus impossible to genotype

beforehand. As a solution, we developed a genotyping assay using asymmetrical PCR combined with unlabeled probe melt analysis (Figure 4.3). Targeting the same short amplicon as above, asymmetrical PCR containing an excess of forward primer and a limited amount of reverse primer on the opposite strand were performed in the presence of LCGreen and a 3' phosphorylated oligonucleotide probe. This results in a major product that is complementary to the excess primer. A 30 bp probe that is complimentary to the TALEN-targeted region of the major product, but cannot participate in the PCR reaction due to the 3' modification which serves as a binding partner during melt analysis. The probe either forms a perfect match, in the case of a wild type allele, or a mismatch duplex, which can be readily distinguished on the melt curve.

We attempted to use TALENs to induce somatic locations in *il1β* by targeting the beginning of exon 3 following the same TALEN procedures as above. HRMA of G_0 embryos indicated that 88% possessed mutations in the TALEN-targeted region. However, when individuals from the G_0 population were outcrossed to wild type fish, we observed that only 13% of G_1 individuals showed evidence of mutant allele transmission via the germline. As a result of this low germline transmission rate, we were unable to isolate a frame shifting indel in the *il1β* locus, despite screening through >50 G_1 adults.

Use of the Tol2kit to generate heat-inducible IL-1β and IL-10 transgenic zebrafish lines

To generate zebrafish lines where expression levels of IL-1β and IL-10 can be modulated, we used the Tol2kit developed in Kwan *et al*. (2007). The Tol2kit allows for easy vector construction and through the To2 transposon features a high efficiency of transgenesis and integration into the germline. Vector construction was completed using the three-insert multisite Gateway system (Thermo Fisher Scientific), where site-specific *att* sites from lambda phage make it possible to directionally combine multiple fragments or entry clones into a destination vector. To mediate inducible gene expression, 1.5 kb of the zebrafish *hsp70l* promoter was combined with a Kozak sequence, the mRNA sequence of *il1β* or *il10*, and a polyA tail. To be able to visualize the intensity and location of transgene expression, we added *mCherry* connected by a 2A peptide self-cleaving peptide, which allows for multiple gene products from a single vector to the 3' end of the expression construct. The *hsp70:IL1β-2A-mCherry* and *hsp70:IL10-2A-mCherry* expression constructs were recombined into the pDestTol2CG2, which features flanking Tol2 transposon ends and an additional *cmcl2:EGFP-pA* cassette which drives EGFP expression in the developing heart and functions as a transgene marker (Figure 4.4A).

To generate the transgenic lines, plasmid DNA for *pDestTol2CG2(hsp70:IL1β-2A-mCherry)* and *pDestTol2CG2(hsp70:IL10-2A-mCherry)* was injected into the cell of one-cell stage embryos along with

transposase RNA to catalyze transgene insertion. Embryos were visually screened for the presence of EGFP expression in the heart at 48 hpf and those displaying green hearts were raised to adulthood. The G_0 adults were then outcrossed to wild type fish to confirm germline transmission.

Validation of *Tg(hsp70:IL1β-2A-mCherry)* and *Tg(hsp70:IL10-2A-mCherry)* lines

Zebrafish are typically maintained at 28.5°C. To functionally confirm our *Tg(hsp70: IL1β-2A-mCherry)* and *Tg(hsp70:IL10-2A-mCherry)* lines, we placed 2 dpf G_0 embryos at 40°C for 30 minutes to stimulate heat-inducible gene expression. After two 30-minute heat shocks in an 8-hour period, we could see significant mCherry expression in *Tg(hsp70: IL1β-2A-mCherry)* (Figure 4.4B) and *Tg(hsp70:IL10-2A-mCherry)* fish (data not shown) at 1 hour following the second heat shock. We also demonstrated an increase in *il1β* transcript levels 1 hour post-heat shock in *Tg(hsp70:IL1β-2A-mCherry)* embryos using qRT-PCR (Figure 4.4C). Notably, at 12 hours after heat shock *il1β* levels had returned to a basal level, indicating that heat-induced expression was transient.

Subsequent generations of the *Tg(hsp70:IL10-2A-mCherry)* fish displayed mosaic *cmcl2*:EGFP expression and significantly reduced mCherry expression, suggesting silencing of the Tol2 insertion site. Despite mCherry expression being difficult to discern by eye in 2 dpf embryos following a 6-hour heat shock at 37°C, immunoblotting indicated that levels of IL-10 protein were increased 1.85-fold (Figure 4.4D). Notably, when wild type embryos were heat shocked under

identical conditions, we saw a 50% reduction in IL-10 levels (Figure 4.4D), indicating that heat shock alone causes a reduction in IL-10 levels and offering an explanation for the lower than expected levels of IL-10 and mCherry expression in *Tg(hsp70:IL10-2A-mCherry)* fish.

Discussion

In this chapter, I described the generation of novel zebrafish lines to study the biology of IL-1β and IL-10 during infection, inflammation, and other biological processes. Using TALENs, I created two mutant lines carrying null mutations in IL-10 and subsequently developed a robust and sensitive protocol for genotyping these lines using asymmetrical PCR combined with HRMA using a 3'-blocked unlabeled probe. Using the Tol2kit, I generated two transgenic lines that place IL-1β and IL-10 under inducible control and provide a mCherry marker for their expression.

The IL-10 deficient fish will be a valuable tool for the growing community of researchers using zebrafish as a model of inflammation and host-microbe interactions. Within my own research field, I think these fish will prove informative in understanding the complicated role of IL-10 during sepsis. Additionally, given the absence of an adaptive immune system in zebrafish embryos, these fish can help parse IL-10's role on the innate immune system from its effects on the adaptive immune system. Considering that genetically distinct *E. coli* strains induce divergent levels of *il10* (Figure 2.11A), examining these infections in an IL-10 deficient background and exploring how this affects the behavior of both the

host and the pathogen will also be interesting. IL-10 is important for the resolution phase of acute inflammation, but also impairs regeneration following injury [13, 50]. The zebrafish has a unique ability to regenerate many of its tissues compared to other vertebrates [51, 52], so the IL-10 deficient fish is well suited for determining the specific contexts and cues by which IL-10 impairs regeneration. Finally, considering the ease of generating and maintaining gnotobiotic zebrafish [53] and the established role of IL-10 in educating the immune system to the resident enteric microbiota, the IL-10 deficient fish will be a valuable tool for studying the establishment and maintence of gut microbial communities, as well as the host immune response to these communities.

Considering that exogenous IL-10 is being explored as therapy for many inflammatory-associated conditions [19], the generation of a zebrafish line where IL-10 is placed under inducible control is a valuable tool. Heat induction was selected as it is commonly used for gene expression in zebrafish and other systems [54]. However, at the time I constructed the lines, I did not appreciate that the use of heat induction in the context of infection and inflammation is problematic. Fever is an evolutionarily conserved response and in ectothermic organisms such as fish, where body temperature is dependent on external sources, a behavioral fever or acute preference for warmer temperatures is often present following infection [54]. Behavioral fever also has a functional benefit following infection. *Carassius auratus* (goldfish) maintained at an increased temperature have a higher survival rate following infection with *Aeromonas*

hydrophila. Behavioral fever also specifically affects the immune response, as demonstrated in *Oncorhynchus mykiss* (rainbow trout). Following injection with LPS, fish show an increased preference for warmer temperatures and notably, possess higher levels of IL-1β at the increased in temperature compared to fish maintained at the homeostatic temperature [55-59]. There is even evidence of heat shock affecting the immune response in zebrafish, where increased neutrophil mobilization but decreased neutrophil recruitment to the site of *Streptococcus iniae* infection or tail injury is seen following heat shock [59]. These muddling effects of heat shock on the immune response, combined with the suboptimal induction of IL-1β and IL-10 requiring extended and repeated heat shocks, have led to limited utility for the *Tg(hsp70:IL1β-2A-mCherry)* and the *Tg(hsp70:IL10-2A-mCherry)* lines.

Materials and Methods

Ethics statement

Animals used in this study were handled in accordance with University of Utah and IACUC-approved protocols following standard guidelines described at www.zfin.org and in the Guide for the Care and Use of Laboratory Animals, 8th Edition.

Zebrafish stock and embryo maintenance

Zebrafish were maintained as breeding colonies on a 14-h/10-h light/dark cycle. Embryos were collected as mixed egg clutches and raised at 28.5°C in E3

medium (5 mM NaCl, 0.27 mM KCl, 0.4 mM $CaCl_2$, 0.16 mM $MgSO_4$; pH 7.4) containing 0.000016% methylene blue as an antifungal agent.

TALEN target site and assembly

The exon sequence for IL10 was scanned for potential TALEN target sites as described in [47]. Once a target site was identified, pCS2TAL3-IL10-TALEN-DD and pCS2TAL3-IL10-TALEN-RR encoding the left and right TALEN, respectively, were constructed by the University of Utah Mutation and Detection Core using a modified TALEN Golden Gate assembly system as in [60].

Injection of TALEN-encoding RNA into zebrafish embryos

5'-capped mRNA encoding the left and right TALEN was generated from NotI-linerized pCS2TAL3-IL10-TALEN-DD and pCS2TAL3-IL10-TALEN-RR by *in vitro* transcription (mMessage mMachine SP6 kit; Invitrogen). mRNA was diluted to 50 ng/μl and 1 nl of this solution injected into one-cell stage embryos.

Genomic DNA extraction

Genomic DNA was extracted from embryos and the fins of adult fish by boiling for 20 min at 95°C in 50 mM NaOH followed by neutralization in 1M Tris-HCl (pH 8.0). Samples obtained from adult fins were then centrifuged at 12,000 rpm for 5 min to remove debris and supernatant transferred to new tubes.

HRMA

HRMA was used to detect TALEN-induced mutations. A 92 bp amplicon surrounding the target site was PCR-amplified in a 10 μl reaction using LCGreen-containing LightScanner Master Mix (Biofire) according to the manufacturer's directions. Melt data were collected on a LightCycler480 (Roche) or LightScanner (Biofire).

Unlabeled 3'-blocked HRMA

Asymmetrical PCR targeting the same genomic region as above and using LightScanner Master Mix was performed using 5 pmol forward primer, 1 pmol reverse primer, and 4 pmol unlabeled probe. Melt data were collected on a LightCycler480 (Roche).

Construction of pME(IL1β) and pME(IL10)

pME entry clones were generated as described in the Invitrogen MultiSite Three-Fragment Gateway manual. Briefly, *att* sites were added to the mRNA sequence of IL-1β (NM_212844.1) or IL-10 (NM_001020785.2) via PCR with Phusion High Fidelity Taq (NEB Biosciences). PCR products were then gel purified and 100 fmoles added to a BP recombination reaction with 150 ng pDONR221. Completed reactions were transformed into DH5alpha cells and resulting colonies prepped and sequence verified using the M13 primer site built into the parent vector.

Construction of *pDestTol2CG2(hsp70:IL1β-2A-mCherry)* and *pDestTol2CG2(hsp70:IL10-2A-mCherry)*

Expression constructs were generated following the protocols described in the Invitrogen MultiSite Three-Fragment Gateway manual. 20 fmol of either *p5E(hsp70v3), pME(il1β),* and *p3E(2A-mCherry)* or *p5E(hsp70v3)*, *pME(il10)*, and *p3E(2A-mCherry)* were combined into an LR recombination reaction with 20 fmol *pDestTol2CG2*. Completed reactions were transformed into OneShot Chemically Competent Top10 and verified by restriction digest.

Injection of *pDestTol2CG2(hsp70:IL1β-2A-mCherry)* and *pDestTol2CG2(hsp70:IL10-2A-mCherry)* into zebrafish embryos

5'-capped transposase RNA was generated from pCS2FA-transposase by *in vitro* transcription (Invitrogen mMessage mMachine SP6 kit). 25 pg transposase RNA and 25 pg plasmid DNA for each expression construct were co-injected into the cell of one-cell stage embryos.

RNA isolation from zebrafish embryos

Pools of 15-20 embryos were manually homogenized in 1 ml QIAzol Lysis Reagent (Qiagen) and total RNA extracted using the Qiagen RNeasy Plus Universal Kit according to the manufacturer's instructions. Genomic DNA was removed by the gDNA Eliminator Solution (Qiagen).

cDNA synthesis and qRT-PCR

cDNA was synthesized from 2 µg of RNA using the SuperScript III First-Strand Synthesis System (Invitrogen) in a 20 µl reaction volume. Following assembly of the master mix according to manufacturer instructions, samples were incubated at 25°C for 5 min, 55°C for 45 min, and 70°C for 15 min. Complimentary RNA was then removed by addition of RNase H (Invitrogen) for 15 min at 37°C.

qRT-PCR was performed on a LightCycler 480 instrument (Roche) following manufacturer recommendations. Cycling parameters were 95°C for 8 min to activate the polymerase followed by 40 cycles of 95°C for 4 sec, 60°C for 6 sec, and 72°C for 6 sec. Fluorescence measurements were taken at the end of each cycle. Melt curve analysis was performed to ensure that no primer dimers were amplified. All reactions were performed in technical duplicate. Sequences for the forward and reverse primers are listed in Table 2.3. Results were normalized to transcript levels of the housekeeping gene *ef1*α (elongation factor 1-alpha) using $\Delta\Delta C_t$ analysis.

Detection of IL-10 protein in zebrafish embryos

Zebrafish embryos were homogenized in PBS using a Bullet Blender Storm 24 (Next Advance). Following BCA to determine protein concentration, samples were diluted accordingly in 5X sample buffer and boiled for 5 min at 95°C. Samples were then immunoblotted against IL-10 (Abbiotec; 1:200) or β-actin (Abcam; 1:1000) as in [47].

Imaging zebrafish embryos

Zebrafish embryos were imaged using a fluorescent Olympus SZX10 stereomicroscope equipped with an Olympus DP72 camera.

Acknowledgements

I am grateful to Timothy Dahlem and the Mutation and Detection Core at the University of Utah for his work in designing and generating the TALEN-encoding plasmids, *pCS2TAL3-IL10-TALEN-DD* and *pCS2TAL3-IL10-TALEN-RR*. I also thank Fabienne Poulin and Kristin Kwan for their advice regarding the design and construction of the Tol2 lines. *p5E-HSv3* and *p3E-2A-mCherry* were kind gifts from Kristin Kwan. *pDestTol2CG2* and *pCS2FA-transposase* was obtained by MTA from Koichi Kawakami.

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Table 4.1. Oligonucleotides used in Chapter 4.

Primer	Sequence (5'-3')
zIL10 HRMA	
Forward	TCTTGTGTGTATTATCAACAGAGTCC
Reverse	AGAGCTGTTGGCAGAATGGT
zIL10 3'-blocked probe	GAGTCCCTATGGATGTCACGTCATGAACGA
zIL10 Sequencing	
Forward	TGGGTGTCTGAAACAGCAGA
Reverse	CACCATATCCCGCTTGAGTT
pME(IL1β)	
Forward (attB1)	GGGGACAAGTTTGTACAAAAAAGCAGGCTCCACCA TGGCATGCGGGCAATATGAAGT
Reverse (attB2)	GGGGACCACTTTGTACAAGAAAGCTGGGTCGATGC GCACTTTATCCTGCAGCTC
pME(IL10)	
Forward (attB1)	GGGGACAAGTTTGTACAAAAAAGCAGGCTCCACCA TGATTTTCTCTGGAGTCATCCTT
Reverse (attB2)	GGGGACCACTTTGTACAAGAAAGCTGGGTCGTGCT TAACCCTCTTTGAGGCT
zIL1β	
Forward	TGGACTTCGCAGCACAAAATG
Reverse	CACTTCACGCTCTTGGATGA
zEF1α	
Forward	TGAGCGTGGTATCACCATTG
Reverse	CAACACCACCAGCAACAATC

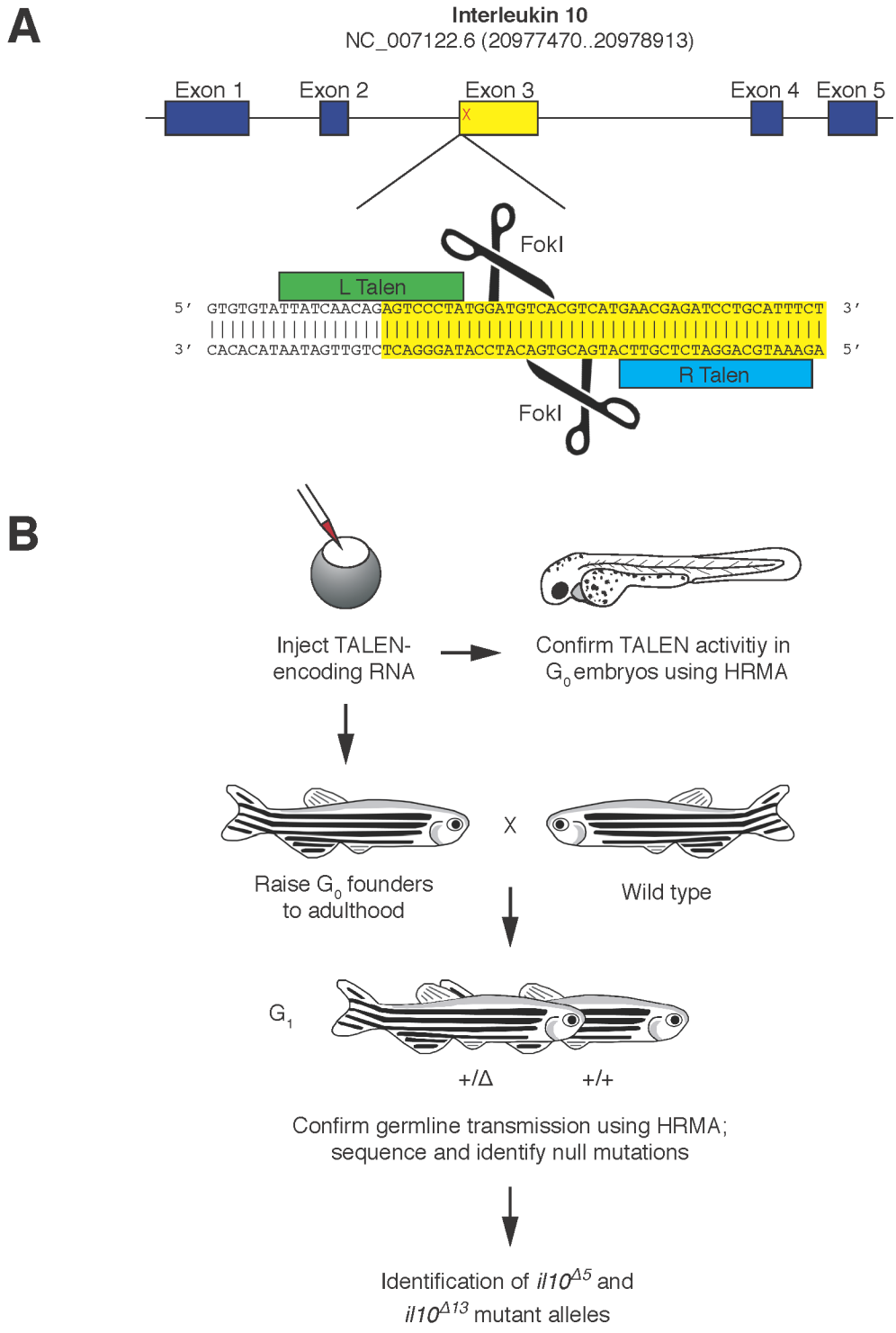
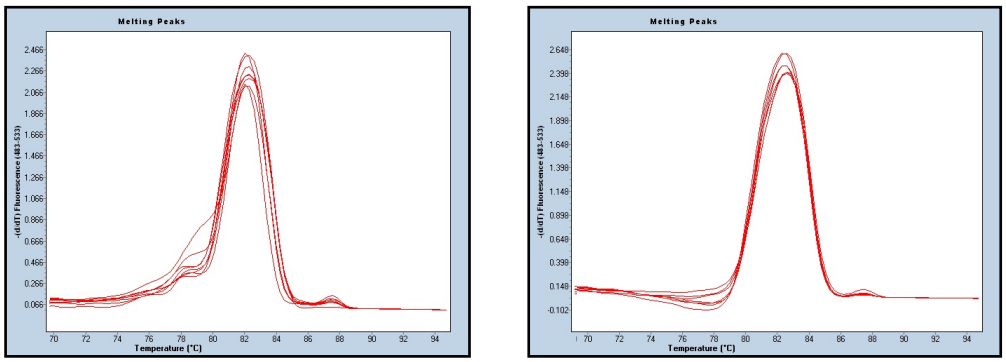


Figure 4.1. Use of TALENs to induce somatic mutations in *il10*.
(**A**) TALENs targeting the beginning of exon 3 were designed to cut and induce somatic mutations in *il10* .

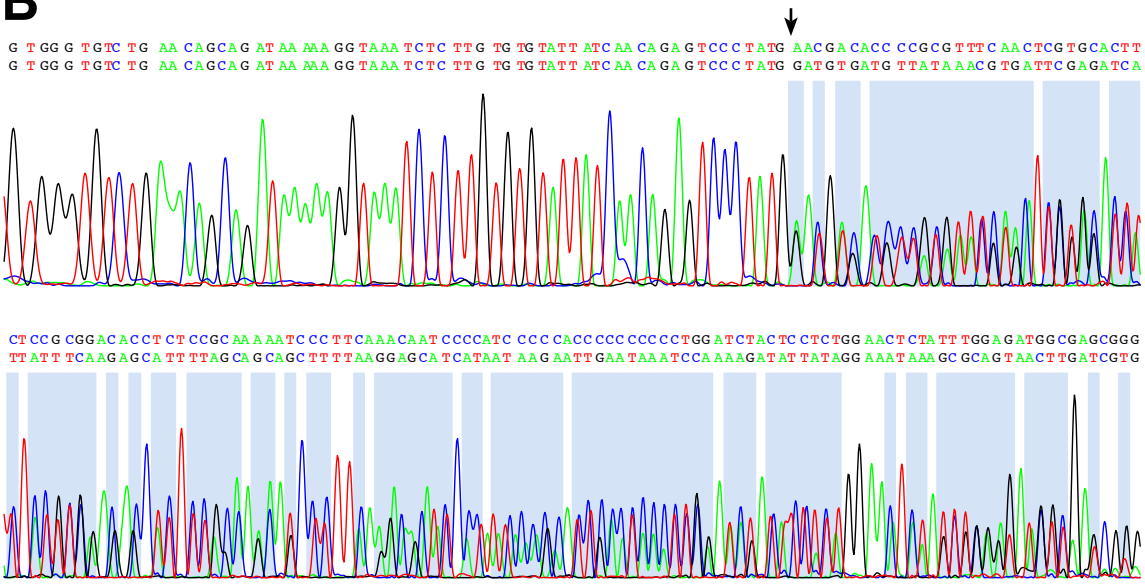
(B) Workflow for generation and identification of the *il10*$^{\Delta 5}$ and *il10*$^{\Delta 13}$ mutant alleles.

A



TALEN-injected embryos Uninjected siblings

B



C

il10	AGTCCCTATGGATGTCACGTCATGAACGAGATCCTGCATTTCT
il10$^{\Delta 5}$	AGTCCCTATGGA-----CGTCATGAACGAGATCCTGCATTTCT
il10$^{\Delta 13}$	AGTCCCTATGGA----------GATCCTGCATTTCT

Figure 4.2. Identification of *il10$^{\Delta 5}$* and *il10$^{\Delta 13}$* alleles.

(**A**) HRMA from G_0 embryos showing differences between the melt curves of TALEN-injected and uninjected siblings, indicating mutations in the targeted region of *il10*.

(**B**) Sanger sequencing chromatogram from a G_1 *il10$^{+/\Delta}$* fish. Multiple peaks appear at the mutation site (arrow) due to the presence of a wild type and mutant allele. The Poly Peak Parser is able to separate this into two separate sequences and identify unknown indels.

(C) Sequence alignment of wild type *il10, il10$^{\Delta 5}$* and *il10$^{\Delta 13}$*.

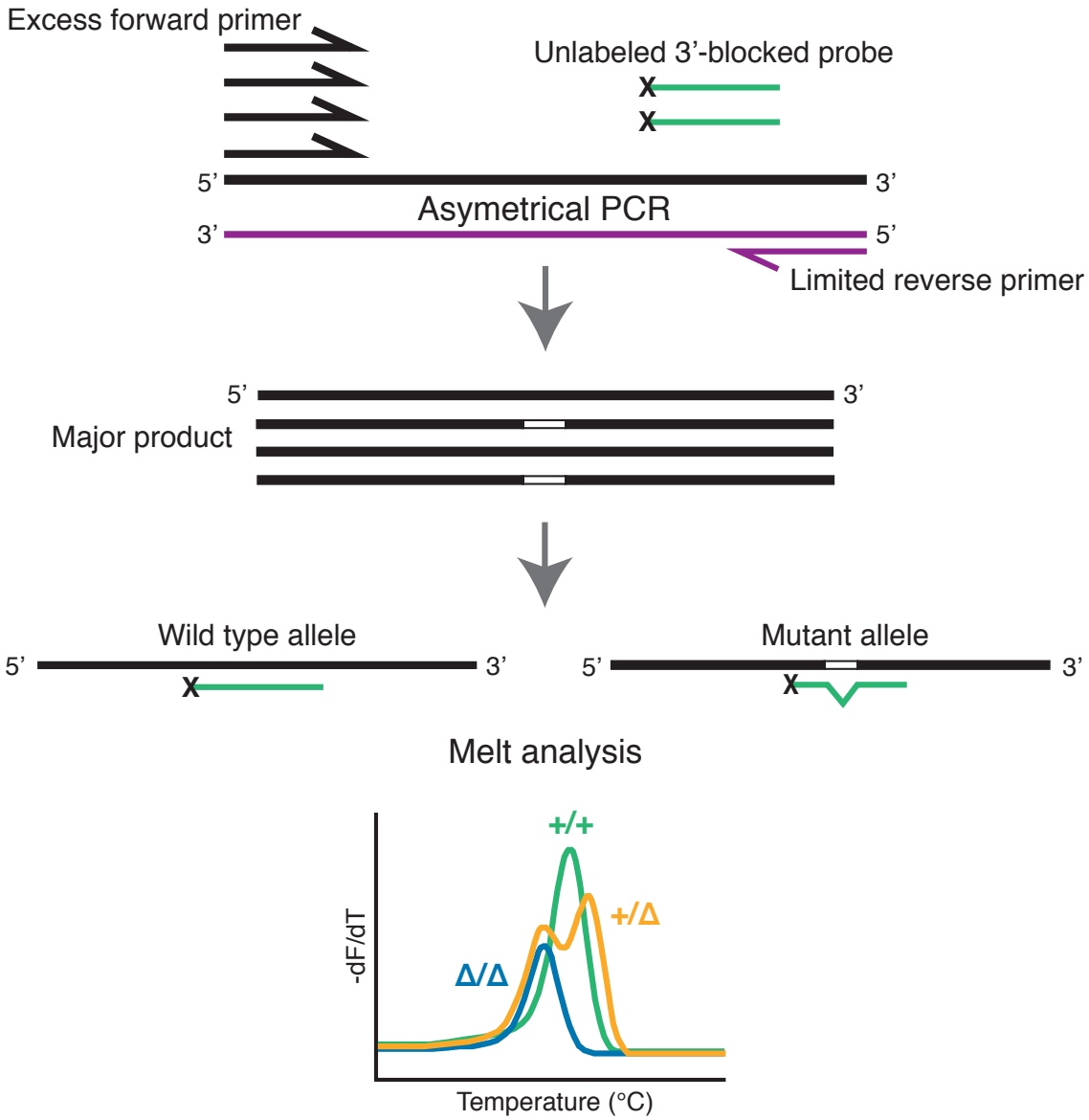


Figure 4.3. Unlabeled probe melt analysis for genotyping of IL10 mutant zebrafish.
Asymetrical PCR containing 5x forward primer, 1x reverse primer, and 4X unlabeled 3'-blocked probe is performed in the presence of LCGreen. The excess of forward primer ensure that the major product will be complementary to the probe, which cannot participate in the reaction due to the 3' modification. During melt analysis, wild type alleles which form a perfect duplex with the probe can be readily distinguished from mutant alleles, which will form a mismatch duplex.

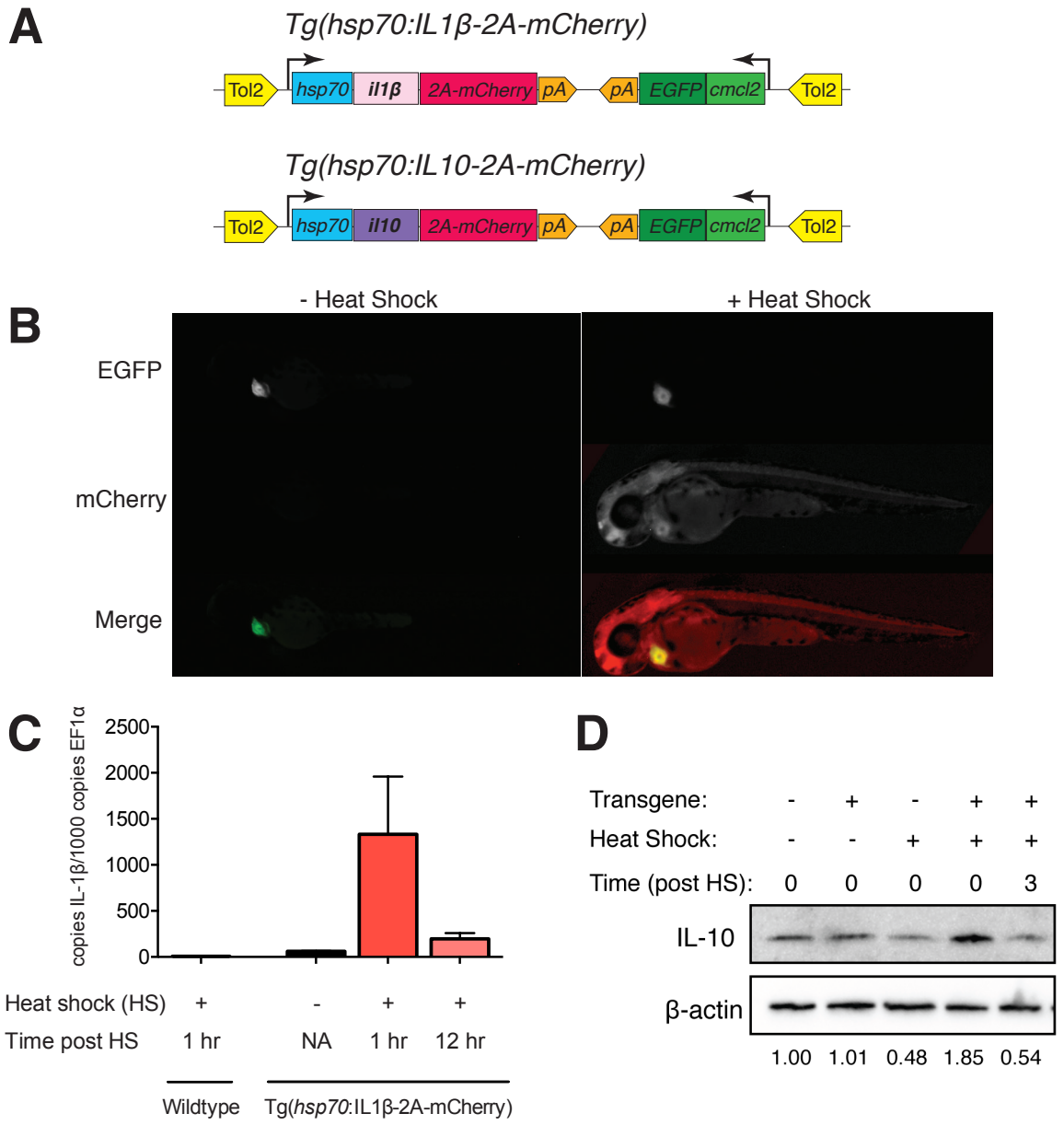


Figure 4.4. Use of the Tol2kit to generate heat-inducible IL-1β and IL-10 transgenic zebrafish lines.

(**A**) Diagram of completed *Tg(hsp70:IL1β-2A-mCherry)* and T*g(hsp70:IL10-2A-mCherry)* expression constructs.

(**B**) Representative fluorescent image of a *Tg(hsp70:IL1β-2A-mCherry)* embryo before and after two 30 min heat shocks at 40°C within an 8-hour period. Images taken shortly after second heat shock.

(**C**) qRT-PCR analysis of *il1β* levels in wild type and *Tg(hsp70:IL1β-2A-mCherry)* embryos. Each bar represents mean results ± SEM from 2 pools of ≥16-20 embryos. qRT-PCR for each pool was carried out using technical duplicates.

(**D**) Immunoblot of IL-10 in wild type and *Tg(hsp70:IL10-2A-mCherry)* embryos following a 6-hour heat shock at 37°C. β-actin blot is provided as a control to show equal protein loading.

CHAPTER 5

DISCUSSION

Sepsis is a life-threatening systemic inflammatory condition. Compounding the burden of sepsis is its high degree of patient heterogeneity, making it notoriously difficult to effectively diagnose and manage. Much of this variability in disease progression has been attributed to differences in host background and immune status. Research presented within this dissertation challenges this belief by demonstrating in a novel zebrafish model of sepsis that genetically distinct *E. coli* strains can trigger divergent host responses, including markedly different levels of inflammatory mediators. These variances correlate with the amount of bacterial flagellin expression during sepsis, as well as differential activation of TLR5 by discrete flagellar serotypes (Figure 5.1). Altogether my doctoral research establishes zebrafish as a relevant model for key aspects of human sepsis and highlights a previously unappreciated ability of genetically distinct *E. coli* strains to induce divergent host responses independent of baseline host attributes, and implicates bacterial flagellin as a key mediator of inflammation during sepsis.

In Chapter 2, I presented the zebrafish as a relevant model for the study of sepsis by demonstrating that infected embryos display signs and pathologies of sepsis, including tachycardia, endothelial leakage, progressive subcutaneous edema, and cutaneous lesions. Embryos also show transcriptional profiles that are equivalent to those seen in human patients, including marked upregulation of pattern recognition receptors, immune signaling cascades, and downstream inflammatory mediators. Reiterating what is seen clinically, timely administration

of antibiotics that curtail bacterial growth within the zebrafish is critical, but only helpful to a point and delays in antibiotic delivery lead to significantly enhanced mortality. My doctoral research also adds new insight to the field's understanding of sepsis, demonstrating that distinct pathologies and divergent host transcriptional responses are seen in response to genetically distinct ExPEC strains. Specifically, I found marked differences in the levels of pro-inflammatory cytokines including IL-1β and IL-8, the antimicrobial peptides Serum amyloid A1 and Hepciden, and also in anti-inflammatory mediators such as IL-10 during infection with these strains. Chapter 2 also presented novel observations regarding the fact that despite similar *in vitro* drug sensitivities, the window during which antibiotic therapy can effectively rescue fish from a lethal infection can vary greatly. Together these findings help explain the problematic variability seen during sepsis and why patients frequently do not present or respond to treatment homogenously.

ExPEC strains often vary by up to 30% of their gene content and have almost unlimited access to new genetic material due to repeated horizontal gene transfer [1-3]. Given this extensive genomic diversity, the results presented here are likely just the tip of the iceberg in terms of how ExPEC strains differentially and uniquely influence the host response during sepsis. For example, the mechanism by which F11 induces the formation of tissue protrusions not observed during infection with CFT073 remains unresolved. Though a number of immunomodulatory genes (e.g., TcpC which antagonizes host TLR signaling and

YbcL which suppresses neutrophil migration [4, 5]) have been characterized in ExPEC strains, there are assuredly myriad other factors that influence the host response within the plastic *E. coli* pan-genome. Given the tractability of zebrafish to forward bacterial genetic screens [6-8], the zebrafish sepsis model has utility in identifying novel ExPEC virulence factors required for survival in the bloodstream during sepsis as well as those that modulate host response(s).

Exploration into the mechanism by which two ExPEC reference strains, CFT073 and F11, trigger distinct host responses in Chapter 3 implicated bacterial flagellin as a key modulator of inflammation during sepsis. This is mediated via variable levels of flagellin expression during infection and the unique observation that distinct flagellar serotypes can differentially activate TLR5. The realization that different versions of flagellin elicit varying host responses suggests that some bacteria may utilize flagella as immunomodulators, expanding their function beyond motility. There are currently 56 characterized H (flagellar) antigens in *E. coli* [9], but the contributions of these different variants to TLR5 activation and downstream host responses remain largely unknown. Additionally, the specific residue or domains that mediate the ability of these flagellin variants to differentially stimulate TLR5 is an interesting and relevant question worth following up on and may open the door to engineered flagellin variants with therapeutic value in regulating host inflammatory responses.

Observations regarding variable levels of IL-1β and IL-10 recorded during sepsis in our model and human patients [10-15] and the lack of available tools to

study these molecules in zebrafish led to the creation of three novel lines. TALENs were used to induce mutations in *il10* and two mutant alleles, *il10*$^{\Delta 5}$ and *il10*$^{\Delta 13}$, were identified to produce an IL-10 deficient zebrafish. Though these lines have not yet been tested phenotypically, I see their utility in further understanding the complicated role of IL-10 during sepsis as well as further delineating the impact of IL-10 on regeneration, colitis, and the microbiota. Despite unsuccessful efforts to generate an IL-1β deficient fish, I still think the creation of this line would be useful given the role of this cytokine in many inflammatory disorders and metastasis.

The findings from my doctoral research (summarized in Figure 5.1) can be applied in the future to better diagnosis and treatment of sepsis. The incidence of sepsis is increasing rapidly and hospitalization rates for sepsis have more than doubled since 2000 [16]. One of the key issues concerning sepsis is the heterogeneity seen in the patient population. Understanding that infections caused by the same bacterial species may present with different pathologies and induce variable host responses will hopefully aid in the earlier diagnosis of sepsis, one of main goals of the “Survive Sepsis Campaign” [17]. Earlier detection of sepsis will help to lower the unacceptably high mortality rate associated with the disease. It will also relieve some of the financial burden placed on the healthcare system by sepsis, where the average sepsis admission costs more than $20,000 and adds up to over $20 billion dollars per year in the US alone [18]. The variability seen in sepsis has resulted in a lack of satisfactory

biomarkers for its diagnosis. Further study on how different microbes contribute to differences in host response during sepsis may lead to the identification of novel panels of biomarkers that are tailored to specific infecting microbes or even subgroups within a single microbial species. The conclusions of my dissertation can also potentially be applied more directly to the treatment of sepsis, where determining the flagellar serotype present during *E. coli* sepsis or assessing levels of flagellin in blood could prove to be a useful tool for informing treatment choices. Finally, the introduction of the zebrafish model for sepsis will also provide a useful platform for the examination of cellular processes during sepsis and the much-needed discovery of improved therapies for sepsis and associated sequelae.

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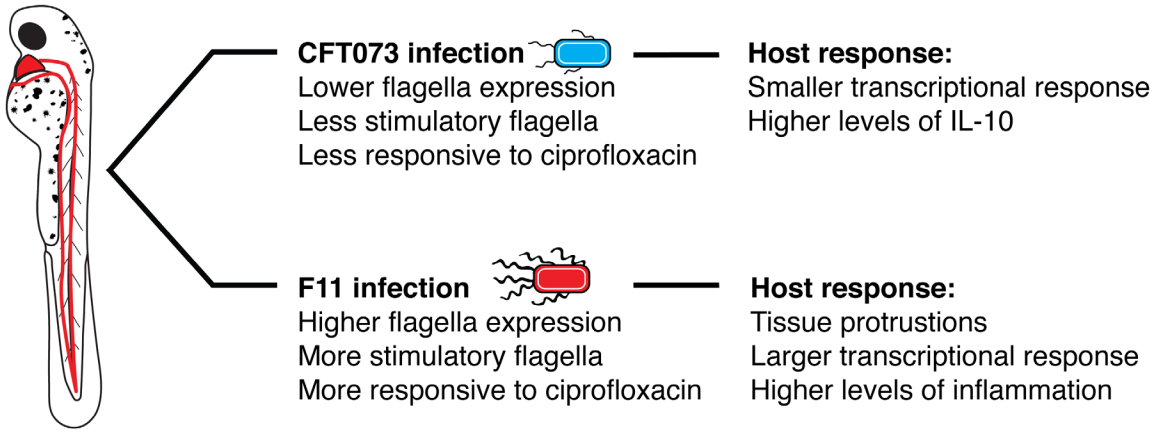

Figure 5.1. Model and summary.
During sepsis, extraintestinal pathogenic *Escherichia coli* strain F11 produces high levels of a very immunostimulatory flagella, leading to a robust transcriptional response and a higher level of inflammatory mediators compared to other E. *coli* strains. Infection with this strain also induced the formation of tissue protrusions and was more responsive to treatment with ciprofloxacin. In contrast, strain CFT03 produces lower levels of a less immunogenic flagella, resulting in a smaller transcriptional response, lower levels of inflammation, and higher levels of IL-10 during sepsis.

APPENDIX A

URINARY TRACT INFECTIONS: CURRENT AND EMERGING MANAGEMENT STRATEGIES

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Barber AE, Norton JP, Spivak AM, Mulvey MA. Urinary tract infections: current and emerging management strategies. *Clinical Infectious Diseases,* 2013;57(5):719-24.
doi: 10.1093/cid/cit284.

INVITED ARTICLE CLINICAL PRACTICE

Ellie J. C. Goldstein, Section Editor

Urinary Tract Infections: Current and Emerging Management Strategies

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Acute cystitis is one of the most commonly encountered bacterial infections and is responsible for substantial morbidity and high medical costs in the United States and across the globe. Though generally considered to be self-limiting and easily treated with antibiotics, urinary tract infections (UTIs) are often incompletely resolved by antibiotic therapy and frequently recur. This is in part due to the ability of uropathogenic bacteria to invade, replicate, and persist within host epithelial cells. The biological complexity of these infections combined with a dramatic rise in antibiotic-resistant pathogens highlight the need for alternative therapies. In this review we examine current management strategies for UTIs, as well as emerging treatments, including novel compounds that block bacterial interactions with the urothelium and vaccines focused on preventing both acute and recurrent infections.

Keywords. UPEC; antibiotic resistance; vaccine; cystitis; recurrent UTI.

A urinary tract infection (UTI) is defined as microbial infiltration of the otherwise sterile urinary tract and is one of the most common bacterial infections worldwide. UTIs encompass infections of the urethra (urethritis), bladder (cystitis), ureters (ureteritis), and kidney (pyelonephritis). There is an estimated annual occurrence of well over 8 million UTIs in the United States, many of which result in a visit to a physician [1]. Nearly all patients with UTI are prescribed a regimen of antibiotics, with roughly 1% of patients requiring hospitalization. The annual cost of UTI treatment in the United States is estimated at $2.14 billion [2], a value compounded by the frequency of recurrent infections. In this review we discuss the epidemiology of acute and recurrent UTIs, detail current management strategies, and explore emerging therapeutics.

Received 14 January 2013; accepted 13 April 2013; electronically published 3 May 2013.

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Clinical Infectious Diseases 2013;57(5):719–24

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DOI: 10.1093/cid/cit284

EPIDEMIOLOGY OF UTIs

UTIs are the most frequent bacterial infection seen in the outpatient setting: 1 in 3 women will develop a UTI requiring antibiotic treatment by age 24, and 50% experience at least 1 UTI during their lifetime [1]. The incidence of cystitis is significantly higher in women than men, likely the result of anatomic differences. Specifically, the shorter female urethra can facilitate bacterial transit from the urethral opening to the bladder. Colonization of the vaginal introitus by gastrointestinal pathogens can also increase the likelihood of urinary tract infiltration [3, 4]. Other factors, including urinary tract obstruction, incomplete voiding, and aberrant structural anatomy also predispose individuals to UTIs. Additional risk factors include prior history of UTIs, vaginal intercourse within the past 2 weeks, use of contraception with spermicide, low vaginal estrogen levels [1, 5], and individual genetic background (extensively reviewed in [6]). While a number of comorbidities increase susceptibility to UTI, the majority of UTIs occur in otherwise healthy women.

The most common bacterial cause of uncomplicated community-acquired UTI is uropathogenic *Escherichia coli* (UPEC), representing >80% of infections [1]. These

bacteria inhabit the lower intestinal tract of warm-blooded vertebrates where they lead a seemingly innocuous existence until they gain access to a niche, such as the urinary tract, where they can cause disease. Other pathogens commonly associated with uncomplicated UTI include *Staphylococcus saprophyticus*, *Klebsiella* species, *Proteus mirabilis*, and *Enterococcus faecalis* [7].

One of the more ominous issues on the horizon for bacterial infections, with UTIs being no exception, is the rise of antibiotic-resistant organisms. One especially troubling example is the heightened incidence of sequence type 131 (ST131) strains of UPEC around the world. These strains often exhibit high levels of resistance to multiple antibiotics and have undergone rapid intercontinental dispersal over the last decade [8]. ST131 strains are an increasingly common cause of community-acquired UTIs, spurring efforts to better identify and treat these resilient pathogens [8, 9]. Factors driving the global spread of ST131 strains are incompletely understood, but likely include the acquisition of antibiotic resistance genes, such as those encoding extended-spectrum β-lactamases (ESBLs), and the capacity to effectively utilize a broad range of metabolites [8, 10]. These characteristics may give ST131 strains a competitive advantage within host environments, increasing the likelihood of their dissemination within and between individuals.

RECURRENT UTIs AND INTRACELLULAR BACTERIAL RESERVOIRS

The burden of UTIs is compounded by their high rate of recurrence. Recurrent UTI (rUTI)—defined as 2 uncomplicated infections in a 6-month time period or 3 infections within a year—cause a tremendous amount of morbidity and are frustrating to patients and physicians alike [1]. Despite administration of antibiotics that seemingly clear the infection (determined by negative urine cultures), the probability that a patient will develop a second UTI within 6 months is 25%, with the chance of recurrence over a 12-month period increasing to 46%. The historical view of rUTI pathogenesis is that each recurrence represents an independent inoculation of the urinary tract. However, this model does not satisfactorily explain many (>50%, by some estimates) rUTI episodes in which the bacterial strains responsible for both the initial infection and the recurrence are genetically identical [11]. An alternative mechanism for recurrence involves the establishment of protected, intracellular bacterial reservoirs within the bladder mucosa (Figure 1).

UPEC can invade host epithelial cells, including the terminally differentiated superficial umbrella cells that line the lumen of the bladder, as well as the underlying, immature

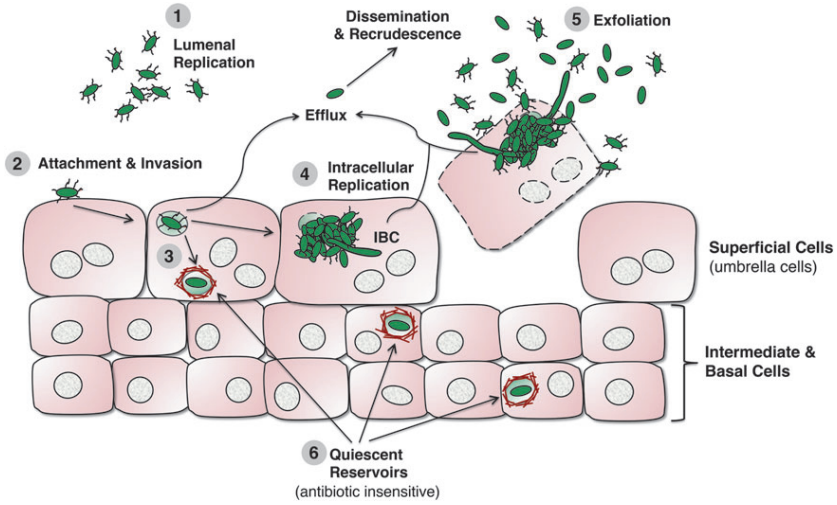

Figure 1. Events that promote the establishment and recurrence of urinary tract infection (UTI). (1) During a UTI, uropathogenic *Escherichia coli* (UPEC; green) can replicate within the lumen of the bladder or (2) attach to and invade bladder epithelial cells. (3) Following invasion, UPEC is either shuttled back out to the lumen or trafficked into late endosome-like compartments. (4) Disruption of these compartments and subsequent entry of UPEC into the host cytosol allows for rapid intracellular bacterial growth and the development of intracellular bacterial community. During these events, UPEC can acquire alternate morphologies, including the formation of long, filamentous cells that are resistant to host defenses such as neutrophils. (5) Infection can trigger the exfoliation of bladder cells, a process that aids in the elimination of adherent and internalized bacteria. The efflux of UPEC from host bladder cells, including those undergoing exfoliation, facilitates pathogen dissemination within and between hosts. (6) UPEC that remains bound within late endosome-like compartments in the urothelium can establish long-lived intracellular quiescent reservoirs that are often enmeshed within actin filaments (red) and extremely difficult to eradicate with antibiotic treatments. The resurgence of UPEC from these reservoirs can initiate recrudescent infections. Abbreviation: IBC, intracellular bacterial community.

intermediate and basal cells [12]. Within superficial bladder cells, UPEC can enter the host cytosol and rapidly multiply, forming a biofilm-like assembly known as an intracellular bacterial community (IBC) [12, 13]. The development of IBCs can enhance the ability of UPEC to establish itself within the urinary tract, building up large numbers of bacteria while sequestered away from the flow of urine and the influx of inflammatory cells and antibacterial molecules. IBCs, however, are not long-lived and will eventually disperse or be shed along with the infected host cells [14]. Indeed, the remnants of IBC-containing host cells can be detected in urine samples isolated from women seeking treatment for UTIs [3]. The efflux of UPEC from within IBCs, as well as the eventual exfoliation of the infected superficial cells, may potentiate the dissemination of UPEC both within the urinary tract and between hosts.

Rather than forming an IBC, UPEC can enter a dormant state within host epithelial cells after trafficking into membrane–bound compartments that become enmeshed within host actin filaments [12]. The quiescent nature and intracellular localization of these bacteria renders them resistant to most antibiotics and inaccessible to infiltrating neutrophils and other host defenses [13, 14]. Experimental models indicate that these quiescent intracellular UPEC reservoirs can persist for long periods in the absence of any overt clinical symptoms, even with the use of antibiotic treatments that effectively sterilize the urine [14]. Environmental signals, such as the reorganization of actin filaments that occurs as bladder cells undergo terminal differentiation, can trigger the resurgent growth of UPEC, prompting the development and dispersal of IBCs and the reinitiation of clinical symptoms. According to this model, rUTIs may in many instances be more accurately defined as recrudescent infections. These issues highlight the need for therapeutic strategies that effectively target both active and dormant stages of UTI.

CURRENT MANAGEMENT OF UTIs

Initial diagnosis of acute uncomplicated cystitis is typically based on patient medical history, taking into account past individual and family health issues, sexual activity, and current symptoms. Common indicators of acute cystitis include urinary urgency and frequency, pain when voiding (dysuria), lower abdominal discomfort, and cloudy or dark urine. The diagnosis of patients presenting with these classic symptoms may be confirmed by urinalysis showing the presence of red blood cells, high nitrite levels, and leukocyte esterase in the urine.

Although medical history and urinalysis are sufficient for the diagnosis of most uncomplicated UTIs, the gold standard for diagnosis of acute cystitis includes a bacteriological urine culture with identification of the causative agent and antimicrobial susceptibility testing. Using fresh, midstream urine, clinical confirmation of an uncomplicated UTI is classically defined as $\geq 10^5$ colony-forming units (CFU)/mL of urine. However, this definition has recently been modified based on observations that many uropathogens are capable of eliciting clinical pathology in the urinary tract even with low levels of bacteriuria [1]. Consequently, as little as 10^3 CFU/mL urine, in the presence of overt UTI symptoms, is now considered sufficient for diagnosis of acute cystitis [15]. Current recommended treatments for acute uncomplicated cystitis are described in Table 1 [1, 16].

Treatment of rUTI

For women who suffer from rUTI, low-dose antibiotic prophylaxis such as nitrofurantoin (100 mg per day), cephalexin (250 mg daily) or trimethoprim-sulfamethoxazole (40 mg/200 mg daily) can provide symptomatic relief and protection against subsequent infections [17]. For women whose UTIs are coincident with sexual activity, a single, postcoital prophylactic antibiotic can be effective in preventing infections [18]. Self-initiated antibiotics are also useful for women with frequent recurrent infections. After diagnosing themselves based on symptoms and/or a urine dipstick, they can initiate a 3-day regimen without needing to visit a physician [18].

The increasing prevalence of antibiotic-resistant uropathogens is likely to limit the effectiveness of our current antibiotic arsenal. For example, individuals who suffer from serious recurrent or chronic UTIs due to ESBL-producing ST131 strains may benefit greatly from carbapenems such as ertapenem [9], but these antibiotics are considered one of our last lines of defense and so should be used cautiously. The ongoing emergence of antibiotic-resistant strains, in conjunction with the high frequency of rUTIs, highlights the need for a better understanding of these infections and the development of new therapeutic strategies.

EMERGING THERAPIES

As noted above, many rUTIs are thought to arise from the ability of bacteria to attach to and invade the bladder mucosa, where they can form intracellular reservoirs protected from antibiotics and host defenses. As such, many emerging treatments for UTIs are aimed at blocking adhesion of bacteria to the urothelium and thereby preventing the establishment of troublesome reservoirs. Type 1 pili (or fimbriae), which are multiprotein filamentous adhesive structures encoded by virtually all UPEC isolates, are generally indispensible for colonization of the urinary tract [11]. The adhesin protein FimH, which is localized at the distal tip of each type 1 pilus, binds mannose residues on host glycoprotein receptors and allows UPEC to adhere to and invade host bladder cells [19]. Type 1 pili also promote biofilm formation and the development of IBCs [20]. Because type 1 pili are important colonization factors, the

Table 1. Common Treatment Options for Uncomplicated Cystitis

Antibiotic	Mechanism	Dosage	Notes
Nitrofurantoin monohydrate/macrocrystals	Inhibits protein, DNA, RNA, and cell wall synthesis	100 mg orally, twice daily for 5 d	Low resistance rates and risk of adverse side effects. Similar efficacy compared to a 3-d regimen of trimethoprim-sulfamethoxazole
Trimethoprim-sulfamethoxazole	Inhibits nucleic acid synthesis by folate synthesis inhibition	160 mg/800 mg (1 double-strength tablet), twice daily for 3 d	Only for use when local resistance rates do not exceed 20% and in patients who do not have sulfa drug allergies
Fosfomycin trometamol	Blocks cell wall synthesis by inactivating enolpyruvyl transferase	3 g in a single dose	Minimal resistance and risk of collateral damage. Inferior efficacy compared to other regimens
Pivmecillinam	Disrupts synthesis of cell wall by inhibiting formation of peptidoglycan cross-links	400 mg, once daily for 3–7 d	Low resistance rates and risk of adverse side effects. Not available in North America

The choice of antibiotics should be made after considering patient allergy and compliance history, local resistance rates, drug availability, and cost. Fluoroquinolones such as ciprofloxacin are highly effective and can be given if none of the recommended antimicrobials can be used. However, resistance rates to these drugs are on the rise and it is recommended that they be reserved for conditions other than acute cystitis. β-lactam antibiotics, such as amoxicillin, cefdinir, cefaclor, or cefpodoximine, in 3- to 7-day treatment regimens can be given when other recommended agents cannot be used. However, β-lactam antibiotics have inferior efficacy and a higher rate of resistance, particularly in ST131 strains. Ampicillin should not be used because it displays relatively poor efficacy in the treatment of urinary tract infections and resistance rates to ampicillin are typically high.

therapeutic potential of inhibiting the assembly or function of these adhesive organelles has received considerable attention.

Pilicides and Mannosides

The assembly of type 1 pili occurs through the chaperone-usher pathway, relying on the periplasmic chaperone FimC for the stabilization, folding, transport, and assembly of pilus subunits [21]. Small synthetic molecules known as pilicides, which are designed to target periplasmic chaperones and consequently interfere with pilus assembly, provide an attractive approach for blocking bacterial adhesion and subsequent reservoir formation. In vitro, pilicides effectively inhibit pilus biogenesis, reducing UPEC adherence to bladder epithelial cells as well as type 1 pili–dependent biofilm formation [22, 23]. The efficacy of pilicides in animal infection models has not been reported.

Researchers have also specifically targeted the FimH adhesin by use of soluble receptor analogues, or mannosides, that act as antiadhesives. These molecules bind FimH and prevent it from interacting with host receptors [24]. Recently, orally available mannoside derivatives have been developed that show great promise as therapeutics [25, 26]. In a murine UTI model, these agents work prophylactically, preventing bacterial invasion into bladder tissue [26]. They can also be used to treat established and catheter-associated infections, acting synergistically with standard antibiotic treatments to reduce UPEC titers within the urinary tract of infected mice [27].

Both mannosides and pilicides have exciting potential as future therapies for the treatment of uncomplicated cystitis and rUTI, and both types of reagents may help circumvent the rising tide of antibiotic-resistant organisms. However, one potential concern with the systemic administration of either mannosides or pilicides is potential adverse effects on commensal *E. coli* strains and other members of the intestinal microbiota, many of which also express type 1 pili [28]. Current thinking by many in the field is that use of pilicides and mannosides will likely be less disruptive than current antibiotic treatment protocols, but this supposition requires additional investigation.

Vaccinology

An alternate strategy for the prevention of recurrent and chronic UTIs is the development of systemic or mucosal vaccines. Over the past 20 years, several vaccination approaches have been explored, including the use of heat-killed whole bacteria, bacterial cell extracts, and purified UPEC-associated virulence factors as antigens. Vaccination of women using a vaginal suppository containing 10 heat-killed strains of uropathogenic bacteria showed much promise in recent years [29, 30]. This multivalent vaccine formulation, known as Solco Urovac, included 6 *E. coli* strains plus 1 strain each of *P. mirabilis*, *Morganella morganii*, *Klebsiella pneumoniae*, and *E. faecalis*. Urovac passed phase 2 clinical trials and was shown to reduce the incidence of UTI caused by *E. coli* in sexually active women

between 20 and 50 years of age with histories of rUTI [29]. Although some individual patients in the study showed increases in anti–*E. coli* antibody levels, no statistically significant differences between vaccinated and placebo control groups were detected, possibly accounting for the lack of any follow-up phase 3 trials.

Specific bacterial factors that have been targeted as vaccine candidates for UTI include the type 1 pilus–associated adhesin FimH and UPEC-associated iron acquisition systems. Like pilicides and mannosides, antibodies directed against FimH can interfere with the functionality of type 1 pili, disrupting the ability of UPEC to colonize the urinary tract. Vaccination with purified FimH coupled to its periplasmic chaperone FimC offered protection against UPEC when administered systemically in both murine and primate models of cystitis [31–33]. A similar vaccine containing a truncated version of FimH protected mice from experimentally induced cystitis when given by either intramuscular or intranasal (mucosal) inoculation, using CpG oligonucleotides as adjuvant [34].

Most bacteria require iron for survival, and while there is ample iron in the human body, it is sequestered and generally inaccessible to bacteria. Consequently, UPEC and many other pathogenic bacteria rely upon iron-chelating molecules and receptors that enable them to scavenge essential iron from the host [35]. Use of purified bacterial iron receptor proteins for vaccination against UPEC has had mixed results. Of 7 UPEC-associated iron receptors tested as vaccines in mice, 2 (IreA and LutA) provided significant protection against experimentally induced cystitis [36]. Vaccination with another iron receptor, Hma, protected against kidney infection, but not cystitis. For this analysis, the purified iron receptors were delivered intranasally after being conjugated to cholera toxin to increase antigenicity.

In total, these studies highlight both FimH and iron receptors as potentially valuable vaccine candidates that merit further investigation. However, as with mannosides and pilicides, the use of purified iron receptors, FimH, or other UPEC-associated factors as vaccines may have inadvertent effects on members of the endogenous microbiota that should be considered. In addition, the route of vaccine delivery and the types of adjuvants utilized need to be optimized for maximal efficacy in humans. While individuals prone to recurrent or chronic UTIs may benefit greatly from the development of anti-UPEC vaccines, the costs and risks of this strategy require further evaluation.

Despite these hurdles, initial success in the development of anti-UPEC vaccines has spurred the search for additional vaccine antigens. Candidate approaches, in which known virulence factors such as flagellin are targeted, continue to generate promising results [37], but less biased methods that are not necessarily reliant on our limited understanding of UTI pathogenesis may prove more fruitful. Along these lines, researchers have developed in silico approaches, known collectively as reverse vaccinology, to probe the increasingly large number of sequenced bacterial genomes for pathogen-specific, surface-localized antigens [38, 39]. These traits in a vaccine antigen should increase the efficacy of antibody responses while limiting cross-reactivity with nonpathogenic bacteria. This approach to vaccine design is encapsulated in a publicly available, Web-based system known as Vaxign (http://www.violinet.org/vaxign/). By screening for outer membrane proteins with amino acid sequences that are conserved among UPEC isolates, but absent from nonpathogenic *E. coli* strains as well as humans and mice, Vaxign identified 22 putative UPEC-specific vaccine targets [40]. Several of these are functionally undefined, and a few are known to be expressed by UPEC during UTI, but to date none have been shown to protect against cystitis. The refinement of reverse vaccinology, coupled with gene expression profiling, proteomic analyses, and emerging high-throughput genetic screens, promises to greatly enhance our ability to identify useful vaccine targets.

CONCLUSIONS

Although UTIs are often considered to be easily managed infections, they remain a huge burden for millions of individuals and our healthcare system. The increasing prevalence of antibiotic resistance among uropathogens presents a major challenge to the clinical management of UTIs. Recurrent infections, including those caused by antibiotic-sensitive pathogens, are exceptionally common and are likely attributable in part to the establishment of recalcitrant intracellular bacterial reservoirs within the bladder mucosa. Eradication of these clinically relevant reservoirs will require a better understanding of the underlying molecular mechanisms that allow for their persistence. The ongoing development of new antimicrobial approaches, such as the use of pilicides and mannosides in conjunction with antibiotics, will provide new treatment options, while the identification of new vaccine candidates and optimized vaccination protocols promises relief to individuals who suffer from recurrent or chronic UTI.

Notes

Financial support. This work was supported by the National Institutes of Health (NIH; grants AI095647, AI090369, and AI088086 to the Mulvey laboratory; NIH Microbial Pathogenesis Training Grant T32 AI055434 to A. E. B.; and NIH Genetics Training Grant T32 GM007464 to J. P. N.).

Potential conflicts of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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APPENDIX B

STRENGTHS AND LIMITATIONS OF MODEL SYSTEMS FOR THE STUDY OF URINARY TRACT INFECTIONS AND RELATED PATHOLOGIES

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Barber AE, Norton JP, Wiles TJ, Mulvey MA. Strengths and Limitations of Model Systems for the Study of Urinary Tract Infections. *Microbiology and Molecular Biology Reviews*. 2015;80(2):351-67. doi: 10.1128/MMBR.00067-15

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Strengths and Limitations of Model Systems for the Study of Urinary Tract Infections and Related Pathologies

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SUMMARY

Urinary tract infections (UTIs) are some of the most common bacterial infections worldwide and are a source of substantial morbidity among otherwise healthy women. UTIs can be caused by a variety of microbes, but the predominant etiologic agent of these infections is uropathogenic *Escherichia coli* (UPEC). An especially troubling feature of UPEC-associated UTIs is their high rate of recurrence. This problem is compounded by the drastic increase in the global incidence of antibiotic-resistant UPEC strains over the past 15 years. The need for more effective treatments for UTIs is driving research aimed at bettering our understanding of the virulence mechanisms and host-pathogen interactions that occur during the course of these infections. Surrogate models of human infection, including cell culture systems and the use of murine, porcine, avian, teleost (zebrafish), and nematode hosts, are being employed to define host and bacterial factors that modulate the pathogenesis of UTIs. These model systems are revealing how UPEC strains can avoid or overcome host defenses and acquire scarce nutrients while also providing insight into the virulence mechanisms used by UPEC within compromised individuals, such as catheterized patients. Here, we summarize our current understanding of UTI pathogenesis while also giving an overview of the model systems used to study the initiation, persistence, and recurrence of UTIs and life-threatening sequelae like urosepsis. Although we focus on UPEC, the experimental systems described here can also provide valuable insight into the disease processes associated with other bacterial pathogens both within the urinary tract and elsewhere within the host.

INTRODUCTION

Urinary tract infections (UTIs) occur when pathogenic microbes colonize typically sterile niches within the urethra (urethritis), bladder (cystitis), ureters (ureteritis), or kidneys (pyelonephritis). These infections are among the most common infections experienced by humans, affecting more than 8 million people annually in the United States with associated costs exceeding $2 billion (1–4). UTIs affect individuals of all age groups and both sexes, although they occur most frequently in otherwise healthy women. It is estimated that one in three women will suffer from a UTI by the age of 24 years, with 50% of women experiencing a UTI at least once during their lifetime (1, 5). Compounding the burden of these infections is their high rate of recurrence. For an individual with a UTI, there is a 25% chance that the infection will recur within 6 months, regardless of antibiotic intervention (2, 5). The probability of recurrence over a 12-month period increases to 46%. Individuals with anatomical abnormalities of the urinary tract and patients with urinary catheters are exceptionally prone to UTIs and may develop complicated chronic infections that can be extremely difficult to eradicate.

Cystitis, which is by far the most common type of UTI, is associated with increased urinary frequency and urgency, pain during urination, cloudy or bloody urine, urine with a strong or foul odor, and discomfort in the pelvic region. A low-grade fever can coincide with cystitis but is more frequently associated with pyelonephritis. A patient suffering from pyelonephritis can have any or all symptoms associated with cystitis in addition to costovertebral flank pain, nausea, and vomiting. Pyelonephritis can also lead to renal scarring and diminished kidney function, particularly in

Citation Barber AE, Norton JP, Wiles TJ, Mulvey MA. XXXX. Strengths and limitations of model systems for the study of urinary tract infections and related pathologies. Microbiol Mol Biol Rev doi:10.1128/MMBR.00067-15.

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young children (6, 7). Episodes in which bacteria disseminate from the kidneys into the bloodstream can be especially serious, resulting in bacteremia and life-threatening sepsis (urosepsis) (4, 8, 9).

Most uncomplicated UTIs are easily treated by using standard prescribed antibiotic regimens, including single-dose treatments with antibiotics like fosfomycin or 3- to 7-day treatments with drugs like nitrofurantoin, trimethoprim-sulfamethoxazole, or pivmecillinam (10, 11). Prophylactic low-dose administration of antibiotics can provide relief and protection for individuals prone to recurrent UTIs (12). However, the effectiveness of nearly all antibiotics used to treat UTIs is declining at an alarming rate as the incidence of antibiotic-resistant bacteria is rapidly increasing across the globe (13–15). To counter the rise of antibiotic-resistant uropathogens, there is a pressing need to better understand the host and bacterial factors that contribute to the establishment, persistence, recurrence, and spread of UTIs.

ETIOLOGY OF UTIs

Strains of uropathogenic *Escherichia coli* (UPEC) are the primary cause of UTIs, being responsible for >80% of uncomplicated community-acquired cystitis and pyelonephritis cases (2, 16). Less common, although still highly problematic, are UTIs caused by *Staphylococcus saprophyticus*, *Klebsiella* spp., *Proteus mirabilis*, *Pseudomonas* spp., and *Enterococcus faecalis* (16, 17). In cases of complicated UTI, occurring in people with anatomical abnormalities or in catheterized patients, the microbial etiology is more diverse. UPEC strains are still the prominent pathogens, but these individuals have a higher prevalence of infections caused by *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Serratia marcescens*, and group B streptococci and an increased susceptibility to fungal infections by *Candida* species. Due to its high prevalence, UPEC has been the focus of most research on UTIs and is discussed in detail here.

UPEC and other *E. coli* isolates comprise a highly heterogenic group of Gram-negative bacteria (18, 19). Commensal *E. coli* strains are commonly found in association with the gastrointestinal tract of warm-blooded vertebrates, where the bacteria can establish mutually beneficial relationships with their hosts, providing necessary vitamins (e.g., vitamin K and B-complex vitamins), promoting gastrointestinal homeostasis, and protecting the gut from pathogens (20–23). Pathogenic *E. coli* variants can cause a wide range of diseases both within and outside the gastrointestinal tract. Diarrheagenic strains, like enterohemorrhagic *E. coli* (best known for the O157:H7 serotypes found in contaminated food) and enteropathogenic *E. coli*, can cause gastroenteritis but do not typically cause disease outside the gastrointestinal tract (24). In contrast, UPEC strains appear to rarely cause problems within the gut but can elicit disease when given access to alternative niches such as the urinary tract or bloodstream. UPEC strains originating from the gut are likely the primary cause of UTIs (25), but the factors that promote UPEC fitness within the intestinal tract are just beginning to come to light (26–28).

The ease with which UPEC isolates acquire resistance to commonly used antibiotics contributes to the widespread success of these pathogens. Of deep concern is the recent and rapid intercontinental dissemination of multidrug-resistant UPEC strains, including many so-called ST131 isolates (29–31). In less than a decade, ST131 isolates have become a major cause of UTIs in many parts of the world, including North America (32, 33). The increasing prevalence of multidrug-resistant UPEC strains within the global community is driving the search for more effective approaches to prevent and treat UTIs, which in turn necessitates the development and use of model systems with which we can discern and mechanistically define bacterial and host factors that impact the infection process.

THE LIFE CYCLE OF UPEC

UPEC typically initiates a UTI by traversing the urethra to reach the bladder (25, 34). The gender bias observed in UTI epidemiology is thought to be in large part attributable to anatomical differences in the male and female genitourinary tracts (2). Specifically, women possess a significantly shorter urethra than men, which greatly reduces the distance that infectious microbes must travel from the urethral opening in order to establish an infection within the bladder. In addition, women can harbor UPEC within the vaginal introitus, which may become contaminated with uropathogens through contact with fecal matter or, in some cases, via sexual interactions (34–36).

Each step leading up to bacterial entry into the urinary tract can be problematic for would-be pathogens. For instance, the natural microbial communities (the microbiota) that persist as commensals within the gastrointestinal tract and vagina can competitively interfere with UPEC survival by limiting space and nutrient availability and by altering host immunity (21, 37, 38). It is probable that phages and toxic products such as colicins produced by commensal organisms also restrict the growth and dissemination of UPEC. Within the vagina, for example, the production of lactic acid and other factors by commensal lactobacilli can damage UPEC strains that are in transit between the intestinal and urinary tracts (39–41). Consequently, a healthy vaginal microbiome can help protect women against ascending UTIs. Interestingly, recent research employing massively parallel sequencing techniques suggests that the urinary tract itself may also be home to a protective microbiota (42).

Once within the urinary tract, UPEC faces a barrage of host defenses and other challenges to its survival (43, 44). Among the most formidable of these is the bulk flow of urine. Shearing forces associated with the release of urine during micturition can dislodge nonadherent and weakly adherent microbes, rinsing bacteria from the body before they have a chance to elicit disease. Incomplete voiding of the bladder greatly increases the risks of bacteriuria and UTI, and these complicated infections are especially problematic for individuals who have anatomical abnormalities or neurogenic bladder due to spinal cord injuries, spina bifida, or other medical conditions (45). Catheterization of these patients is common and can inadvertently facilitate the introduction of uropathogens into the bladder.

In uncomplicated UTIs, uropathogens can resist expulsion from the urinary tract via elaboration of adhesive molecules (adhesins) that mediate bacterial attachment to host cells and extracellular matrix components. Mathematical modeling suggests that bacterial attachment to host tissues within the urinary tract is potentially unnecessary, since small numbers of unbound residual bacteria that remain following micturition should be able to multiply fast enough in urine to continually repopulate the bladder lumen as it repeatedly empties and fills (46). Indeed, some ABU isolates seemingly lack the ability to bind host bladder cells, and yet, they can still persist for extended periods at high levels within

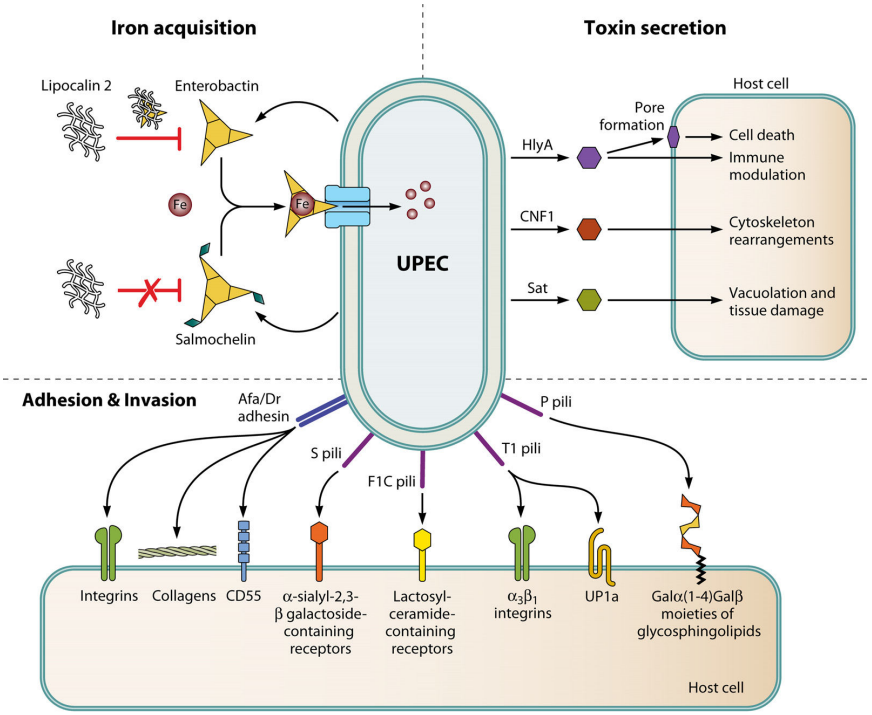

FIG 1 UPEC-associated fitness and virulence determinants. Shown is a sampling of the many different factors that can promote the survival and virulence of UPEC strains within the host. (Left) These factors include iron acquisition systems like enterobactin and salmochelin. Enterobactin is a broadly conserved, high-affinity siderophore that can be inhibited by the host protein lipocalin. To sidestep this host factor, some UPEC isolates produce salmochelin, a glucosylated version of enterobactin that is not recognized by lipocalin. (Right) UPEC strains can secrete a variety of toxins that can disrupt normal host proteolytic cascades, membrane trafficking, cytoskeletal rearrangements, inflammatory responses, and survival pathways. Indicated here are some of the effects of host cell intoxication by alpha-hemolysin (HlyA), secreted autotransporter toxin (Sat), and cytotoxic necrotizing factor 1 (CNF-1). (Bottom) UPEC can bind host cells and tissues via multiple adhesins. These include Afa/Dr adhesins (which bind integrins, collagens, or CD55), S pili (which bind α-sialyl-2,3-β galactoside-containing receptors), F1C pili (which bind lactosylceramide-containing receptors), type 1 pili (T1) (which bind numerous factors, including $\alpha_3\beta_1$ integrins and UP1a), and P pili [which bind Galα(1-4)Galβ moieties of glycosphingolipids].

the urinary tract (47). However, most UPEC isolates appear to require adhesins to effectively colonize the urinary tract.

UPEC isolates encode dozens of adhesins that vary in their receptor specificity and whether or not they are assembled into pili (also known as fimbriae) (34, 48–50) (Fig. 1). One of the most frequently studied adhesins, which is expressed by nearly all UPEC isolates, is the type 1 pilus-associated protein FimH. This adhesin can bind a variety of mannose-containing host membrane glycoproteins as well as components of the extracellular matrix (51). Upon entering the bladder, UPEC encounters a thin glycosaminoglycan-rich glycocalyx that is in loose association with the terminally differentiated superficial umbrella cells that comprise the outer layer of the urothelium (52–54). The apical surface of each umbrella cell is highly enriched for a group of integral membrane glycoproteins known as uroplakins, which are assembled into quasicrystalline arrays of hexagonal complexes (55, 56). Together with underlying layers of less-differentiated epithelial cells, the uroplakin-coated umbrella cells create an exceptionally strong permeability barrier. FimH can mediate bacterial interactions with one of the uroplakins, UP1a, as well as with several other host receptors that may be situated on and within the urothelium (57–62). Although UP1a is doubtlessly the most abundant receptor available to type 1 piliated UPEC on the urothelial surface, other receptors and other adhesins likely come into play as UPEC encounters changing environments and host cell types during the course of infection.

In addition to promoting bacterial attachment to host cells and tissues, several of the adhesins encoded by UPEC isolates can also stimulate the development of biofilm communities. Biofilms consist of bacterial cells encased within a complex extracellular matrix that can include proteins, nucleic acids, and polysaccharides like cellulose. UPEC-associated adhesins that have been implicated in the formation of biofilms include type 1 pili, F1C pili, S pili, and F9 fimbriae as well as the adhesive autotransporter proteins Ag43, UpaG, UpaB, and UpaH (63–70). Flagella and thin aggregative amyloid fibers known as curli can also promote biofilm formation by UPEC (63, 64, 67, 70). The development of biofilms within urinary catheters and in association with host tissues can render UPEC less sensitive to antibiotics and host defenses like neutrophils (70, 71).

The establishment, persistence, and dissemination of UPEC strains within the urinary tract are aided further by the ability of

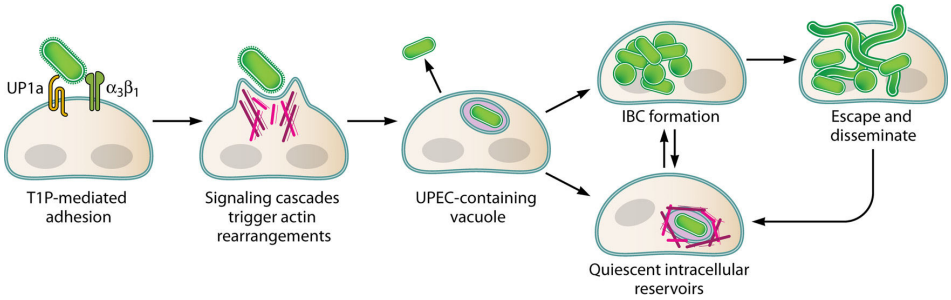

FIG 2 Multiple fates for UPEC in association with the urothelium. Interactions between the type 1 pilus-associated adhesin FimH and the sugar side chains of receptors like UP1a or α3/β1 integrins can stimulate actin cytoskeletal rearrangements leading to the internalization of UPEC. Bacteria are then either trafficked back out of host cells or taken into membrane-bound compartments that are similar to late endosomes or early lysosomes. Within bladder cells, UPEC can remain in a nonreplicating state, forming quiescent reservoirs that are protected from host defenses and antibiotics. Intravacuolar growth of UPEC is restricted in part by the host actin cytoskeleton, which is dense within the immature epithelial cells of the bladder. Within terminally differentiated umbrella cells, where F-actin is localized primarily at basolateral surfaces, UPEC can break into the host cytosol and subsequently multiply to form IBCs. These bacterial communities are not long-lived and are most apparent during the acute phase of a UTI. The disruption or exfoliation of IBC-containing umbrella cells, as well as the transient formation of filamentous bacteria, can facilitate the efflux and dissemination of UPEC. Bacteria released from within IBCs can infect neighboring host cells, including the immature urothelial cells that appear to serve as the primary home for persistent UPEC reservoirs within the bladder. Released bacteria and exfoliated bladder cells that are flushed from the urinary tract with the flow of urine likely facilitate the spread of UPEC between hosts.

these pathogens to invade host epithelial cells (Fig. 2). Among the dozens of adhesins that are encoded by UPEC, type 1 pili and Dr/Afa fimbriae have been implicated most often as mediators of host cell invasion (51). The type 1 pilus adhesin FimH promotes UPEC entry into bladder cells by stimulating actin cytoskeletal rearrangements that cause the host cell membrane to zipper around and envelop adherent bacteria (72). Host receptors for FimH and other UPEC-associated factors that have been implicated as mediators of host cell invasion include integrins (58, 73, 74), the complement regulatory protein CD46 (75, 76), the complement decay-accelerating factor CD55 (77–79), and the flagellin sensor Toll-like receptor 5 (Tlr5) (80, 81). Following entry into host bladder cells, most UPEC cells face one of three fates: (i) they can be quickly returned back to the extracellular environment (82, 83), (ii) they can enter into a nonreplicating quiescent state (83–85), or (iii) they can multiply, forming large intracellular bacterial communities (IBCs) (83, 86, 87). The superficial umbrella cells of the bladder are the initial targets of host cell invasion by UPEC, but the exfoliation of these cells and the influx of neutrophils during the course of a UTI can provide UPEC with access to deeper layers of immature host cells within the urothelium (83, 88, 89).

Within both mature and immature bladder epithelial cells, internalized bacteria that are not immediately expelled are trafficked into membrane-bound compartments that have characteristics of late endosomes and early lysosomes (84, 85, 90). Specifically, these UPEC-containing vacuoles incorporate the host proteins CD63 and LAMP-1 and the lipid lysobisphosphatidic acid (LBPA) but lack the protease cathepsin-D. It is within these compartments, enmeshed within host actin filaments, that UPEC may persist in a nonreplicating state for indeterminate periods that can last for several hours to weeks and perhaps longer (83, 84, 91). Ongoing research indicates that these quiescent intracellular bacteria may serve as reservoirs for the recurrent, or relapsing, UTIs that plague many individuals despite the use of antibiotics (89). Intracellular pathogens are protected from antibiotics due to the permeability barrier function of the urothelium and the inability of many antibiotics to effectively cross host membranes (92). The quiescent bacteria within bladder epithelial cells are also likely more tolerant of antibiotics since most antibiotics affect only replicating microbes.

Changes in both host cholesterol levels and the actin cytoskeleton can stimulate the transition of internalized UPEC from a quiescent state to a rapidly dividing pool of microbes within bladder cells, forming IBCs that can contain several thousand bacterial cells (84, 93). Bacteria present within IBCs are protected from infiltrating phagocytes and are possibly less sensitive to antibiotic treatments (87). During IBC development, many of the normally rod-shaped bacteria transiently assume a filamentous morphology, with some attaining lengths of well over 150 μm (83, 94). These filamentous bacteria are resistant to phagocytosis by infiltrating neutrophils and can extend for long distances within, and even between, host cells. IBCs likely do not serve as long-lived reservoirs for UPEC within the urinary tract, as host cells containing IBCs will eventually rupture or are shed and cleared with the flow of urine (83, 87, 95). The release of UPEC from IBCs as filaments or as single rods can promote pathogen spread both within the urinary tract and, potentially, between hosts. Cycles in which host cell invasion by UPEC is followed by stages of IBC formation, bacterial quiescence, and resurgence may help drive the development of chronic and recurrent UTIs.

In addition to adhesins, UPEC survival within the urinary tract is also facilitated by the expression of various toxins, chelators, and other factors that allow the bacteria to sequester nutrients and essential metals away from the host, modulate host immune responses, and disrupt tissue barriers (a few examples are shown in Fig. 1) (96–99). However, no single set of virulence factors that is required by all UPEC isolates to cause disease has been identified. Rather, UPEC strains appear to rely upon an assorted array of factors that may act redundantly, dependent upon the niche occupied and the specific environmental stresses present. Although the life cycle of UPEC is not fully understood, it almost certainly involves bacterial exposure to various environmental stresses as the pathogens traverse niches both within and between human

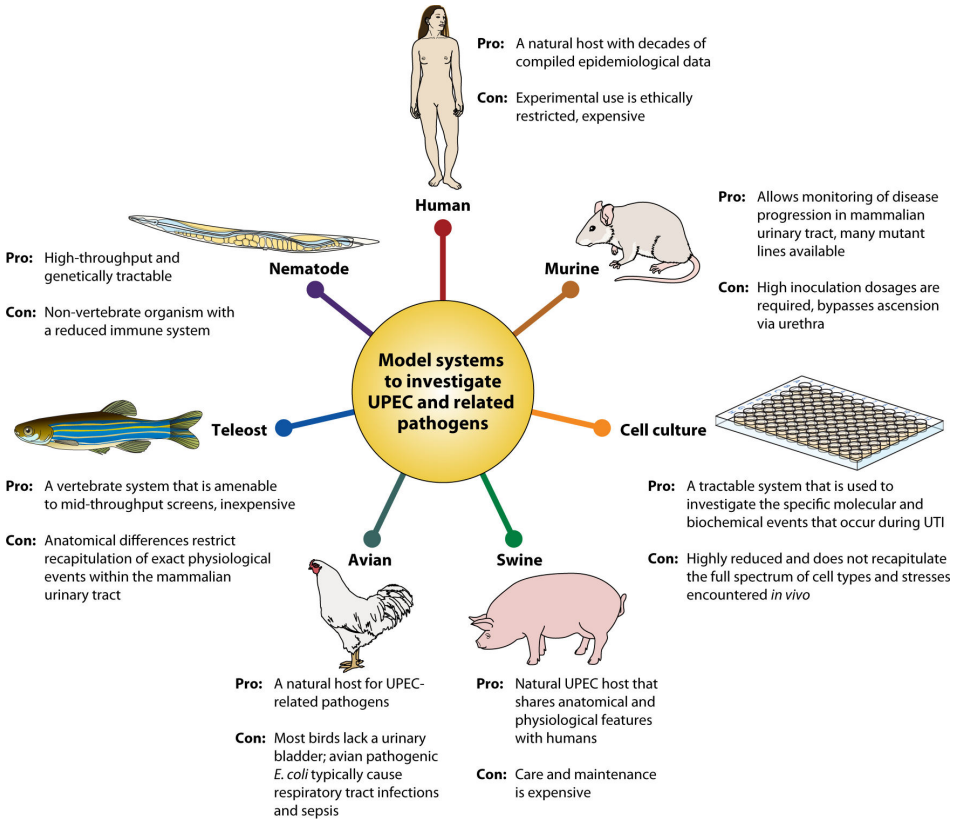

FIG 3 Model systems for investigating the pathogenesis of UPEC and related pathogens. Some of the key pros and cons for each system are indicated.

and animal hosts. For example, UPEC hosts may include pets and farmed poultry and pigs, with time spent between hosts in soil or wastewater or in the meat department of the local grocery store (100–110). The capacity of UPEC and related pathogens to inhabit such a diverse range of niches has helped prompt researchers to develop a variety of model systems to understand the virulence potential, adaptability, and evolution of these ubiquitous pathogens.

MODEL SYSTEMS FOR DEFINING THE PATHOGENICITY OF UPEC AND RELATED BACTERIA

Model systems employed to study the pathogenic behaviors of UPEC isolates have incorporated the use of humans and other primates, rodents, pigs, chickens, zebrafish, and nematodes in addition to primary and cancer cell lines derived from the bladder, kidneys, or other host tissues (Fig. 3). These model systems act as approximations of natural human infections and allow various aspects of the infectious process to be dissected in controlled settings. Thanks to advances in sequencing technology and molecular biology techniques, the genomes of multiple UPEC strains are known and can be manipulated fairly easily by using molecular genetic approaches to generate isogenic mutants and recombinant strains. Pairing molecular and genetic techniques with model systems allows researchers to delineate key bacterial and host factors involved in the pathobiology of UTIs, with a major goal being the identification of novel therapeutic targets. Although our focus here is on UPEC, it should be noted that many of the model systems discussed in the following sections have also been valuable for analyzing bacterium-host interactions involving a diverse array of other uropathogens (for a few of many examples, see references 111–117).

Humans and Other Primates

To date, there have been few experimental UTI studies carried out in humans or other primates. This is in large part due to ethical concerns and the high costs associated with studies in which primates or human volunteers are deliberately infected. Primates are generally employed as part of preclinical trials aimed at testing the efficacy and safety of vaccine and drug candidates. Human studies often involve epidemiological analyses, with the primary aims frequently being to correlate UPEC strain characteristics with patient history and disease information. These studies can be either retrospective or prospective, often centered on the phenotypic and genotypic analysis of bacteria recovered from urine samples provided by human volunteers as well as genetic analysis of the hosts themselves. This sort of work has highlighted the increasing prev-

alence of multidrug-resistant isolates such as ST131 strains and revealed emergent patterns in the types of antibiotic resistance genes and virulence factors carried by UPEC isolates (32, 118–126). For example, analysis of bacterial isolates recovered from the urine of children with febrile UTI indicated that UPEC strains that express type 1 pili cause more severe clinical symptoms than pathogens lacking these adhesins (127). These findings were subsequently verified in mouse and cell culture-based infection models (128, 129).

More recently, techniques like transcriptome sequencing (RNA-seq) have allowed researchers to obtain transcriptional profiles of UPEC isolates recovered directly from the urine of human patients with UTIs. This work indicated important roles for ion transport systems and anaerobic metabolic processes in UPEC within human urine but was limited in its ability to analyze tissue-associated pathogens (130). Still, the results suggested a number of bacterial factors that may be of value as therapeutic targets. Specifically, it was noted that a copper efflux system was highly upregulated in UPEC isolates from human urine, spurring follow-up experiments in which it was shown that copper supplementation can significantly decrease UPEC colonization in a mouse UTI model (130).

Analysis of human-derived samples is especially useful as a means to validate and expand observations made in other experimental systems. For instance, careful microscopic examination of urothelial cells that are shed by human patients during the course of a UTI indicates that the ability of UPEC to invade and multiply within host cells is not a mouse- or cell culture-specific phenomenon, as some have argued (131–135). Instead, the capacity of UPEC strains to act as facultative intracellular pathogens likely contributes to their success as human pathogens. The imaging of UPEC in association with intact human bladder tissue is more problematic, as this requires the acquisition of biopsy specimens or whole bladders, which are not easily obtained.

Taking a more host-centric approach, population-based studies have revealed behavioral, situational, and genetic factors that influence host susceptibility to UTI. Some of the key groups identified as being at greater risk for developing UTI include sexually active females, pregnant women, elderly individuals, and catheterized patients (2, 136). In sexually active women, the use of spermicide or antibiotics escalates the risk of UTI, with the latter also increasing the incidence of infections due to drug-resistant pathogens (137–140). Both spermicide use and antibiotics can disrupt the vaginal microbiota, which normally protects against the ascension of uropathogens into the urinary tract (2, 37, 141). Shifts in the composition of the vaginal microbiota may also account for the increased frequency of UTIs in elderly women (2, 142). This phenomenon is in part attributable to the diminishment of estrogen levels that occurs as women age. Pregnancy substantially increases the risk of pyelonephritis but has little effect on the risk of cystitis. Although pregnancy can alter vaginal secretions and the microbiota (143), the elevated risk of pyelonephritis in pregnant women is more likely attributable to pregnancy-induced increases in blood volume and glomerular filtration rates and the decreased contractility of the ureteric smooth muscle (2). These physiological changes create an environment within the urinary tract that is more conducive to the dissemination and growth of uropathogens. UTI during pregnancy can have serious repercussions, contributing to premature birth, low birth weight, preeclampsia, and the need for cesarean delivery (144). In light of these correlations, researchers are developing mouse models to better understand the mechanisms by which localized UTIs can influence intrauterine fetal growth and other adverse pregnancy outcomes (145, 146).

Specific genetic variables that alter host susceptibility to UTI have been more difficult to nail down, although their existence is suggested by the fact that women with a predisposition for recurrent UTIs often have family members with the same problem (2, 44, 147). Susceptibility factors for pyelonephritis include reduced expression levels of interferon regulatory factor 3 (Irf3) or of the chemokine receptor CXCR1 (147). Polymorphic variants of other surface molecules, including certain blood group antigens and the pattern recognition receptors Tlr4 and Tlr5, may also constitute risk factors, as they can serve as attachment sites for uropathogens or as regulators of host inflammatory responses (147–151). The effects of these and other identified risk factors can be subtle and may not be evident in all studies (152). It is likely that the spectrum of genetic modifiers of UTI susceptibility will continue to expand as researchers learn more about the pathogenic mechanisms and host defenses associated with these infections.

The added complexity of UTI research using epidemiological approaches and samples from human volunteers is assuaged by the opportunity to assess pathogenic processes in a natural environment of direct medical importance. However, this work is not without its drawbacks, which may include, in addition to high costs, the use of avirulent isolates rather than full-fledged pathogens for deliberate infections and the genetic heterogeneity of human volunteers. Consequently, in order to better define the molecular mechanisms of UTI in a more controlled setting, many scientists employ surrogate infection models, keeping in mind how their findings may relate to the situation in human patients.

Cell Culture

Cell culture-based assays have been used extensively to investigate the mechanisms and consequences of UPEC interactions with host cells. These assays typically utilize primary, immortalized, or cancer cell lines derived from the bladder urothelium or from the kidneys (e.g., proximal tubule epithelial cells). Host bladder cells are usually grown as undifferentiated monolayers but can be induced to stratify and partially differentiate when grown with appropriate media on membrane scaffolds in dishes or under microgravity conditions generated within rotary walled vessel bioreactors (153–155). Recently, culture systems that incorporate different types of host cells, such as neutrophils plus bladder epithelial cells, have been established to better approximate the cellular complexity of the inflamed urothelium (156). By using small interfering RNA (siRNA) as well as plasmid and viral constructs, host genes within cultured cells can be specifically silenced or, alternatively, overexpressed, facilitating the functional analysis of host factors that may contribute to the pathogenesis of UTIs (58, 90, 157–159). Pharmacological reagents can also be used to interfere with host enzymes and signaling cascades, but consideration should always be given to dosage and off-target effects as well as potential interference with bacterial processes. Emerging technologies, such as engineered clustered regularly interspaced short palindromic repeat (CRISPR)-Cas9 systems, will allow easier disruption and high-throughput functional analyses of specific genetic loci within host cells (160, 161).

The use of cultured eukaryotic cells has been instrumental in the identification of host receptors that are bound by UPEC as well as

the delineation of downstream signaling events that modulate inflammation and the internalization and trafficking of bacteria (58, 72, 77, 90, 129, 157–159, 162–171). Cell culture-based assays are also commonly used to define how secreted toxins and other UPEC-associated virulence factors affect host cell functions and viability (172–180). The ease of use, low cost, and amenability to high-throughput assays, genetic manipulation, and biochemical analysis make cell culture-based systems attractive alternatives to many animal infection models. Recent advances with flow cell technology even make it possible to examine UPEC-host interactions using bladder cell monolayers in the presence of urine flow, mimicking conditions within the urinary tract during an actual UTI (181).

Despite the proven utility of cell culture-based model systems, they cannot fully recapitulate the complexity of the host environment with its myriad cell types, complicated tissue architecture, variable nutrient levels, and teeming host defenses. For example, key features of terminally differentiated superficial umbrella cells, including their multiple nuclei and quasicrystalline arrays of apical uroplakin complexes, are exceptionally difficult to mimic in cell culture, although notable progress in this area has been made with primary urothelial cells in recent years (154, 182). Therefore, detailing the molecular consequences of UPEC interactions with bladder umbrella cells is currently limited in the context of cell culture-based models. Nonetheless, cell culture models continue to expand our understanding of UTI pathogenesis, oftentimes propelling more insightful follow-up studies in humans and animal model systems.

Murine

The murine UTI model system, which is the predominant animal model for the study of UTIs, usually entails the transurethral catheterization of rodents and subsequent instillation of bacteria directly into the bladder lumen. For consistent colonization between animals, researchers will typically inoculate animals with $\geq 10^7$ CFU, which is likely far higher than the titers that would be present at the onset of a natural UTI. The mouse model is exceptionally pliable and can be modified to assess polymicrobial infections as well as catheter-associated UTIs and chronic cystitis (146, 183–188). Rodents share much in common with humans, including conserved immunological factors and similar anatomical features within the urinary tract (183). While direct instillation of bacteria into the bladder bypasses some steps required during natural infections, this model system still allows detailed investigation of many key aspects of ascending UTIs. Specifically, rodent models have been used extensively for comparative analysis of the fitness and virulence potentials of wild-type and mutant UPEC strains as well as for the examination of host responses to UTI. Bacterial titers associated with the bladder or kidneys can be monitored by plating of tissue homogenates collected at various time points postinoculation or by the use of luminescent bacteria that can be visualized in live animals using biophotonic imaging (189–191). Host responses can be examined by various means, including transcriptional profiling and histological analysis of tissue sections. The use of inbred mouse strains reduces experimental noise arising from host genetic variability, and recombinant genetic engineering technologies allow the generation of whole-animal and tissue-specific gene knockouts.

While the pathogenesis of UTIs in female mice has been studied extensively, relatively little work has been done to understand the impact of these infections on males. This disparity echoes the unequal effects that UTIs have on females in the human population, in which nearly half of all women will endure at least one UTI, compared with only about 12% of males (1). The longer male urethra is thought to hinder bacterial ascension into the bladder, and in the laboratory, this feature renders male mice technically much more difficult to catheterize. One way around this hurdle is to bypass the need for catheterization by instilling bacteria directly into the bladder lumen by way of surgical inoculation via an incision in the abdominal wall (192). By using this approach, it was recently determined that IBC formation and other key events that occur during the development of cystitis are quite similar in males and females. However, notable differences between males and females were observed at later time points. Specifically, male mice had a much higher incidence of chronic cystitis, defined as persistent bacteriuria and protracted inflammation, and were more likely to develop severe pyelonephritis and renal abscesses. These effects were linked with the higher testosterone levels present in male mice, although the mechanisms by which this androgen fuels UTI progression remain unclear (192). Interestingly, older studies indicate that testosterone promotes the secretion of lysosomal hydrolases into the urine (193, 194), a phenomenon that could potentially affect host defenses, including the barrier function of the urothelium.

The availability of genetically distinct mouse strains as well as many mutant knockout and transgenic mouse lines has enabled researchers to assess the specific impact of numerous host genes and immune cells on the progression and outcome of UTIs (44, 195, 196). Mouse lines that vary in their susceptibility to UTI have helped identify host susceptibility factors and provide a platform for assessing the functionality of bacterial fitness determinants. Consider, for example, a recent analysis of isogenic UPEC mutants that lack the *cya* or *crp* gene (197). These genes enable UPEC to synthesize and respond to the second messenger cyclic AMP (cAMP). *In vitro*, the UPEC cystitis isolate UTI89 requires both *cya* and *crp* to effectively utilize alternate carbon sources like amino acids, the principal nutrients available to UPEC within the urinary tract (96, 197, 198). In broth culture assays, the *cya* and *crp* mutants also have increased sensitivities to the damaging effects of reactive nitrogen species and superoxide radicals, akin to those generated by host inflammatory cells (197). In wild-type mice, the *cya* and *crp* mutants are each impaired in their ability to colonize the bladder, whereas in C3H/HeJ mice, the mutants are no longer defective. Due to mutations in the pattern recognition receptor Tlr4 and possibly other immune regulators, C3H/HeJ mice are hyporesponsive to lipopolysaccharide and therefore have very limited inflammatory responses during experimental UTI (196, 199–201). Together, these observations indicate that Tlr4-dependent innate host defenses within wild-type mice, and not nutrient availability *per se*, are the major factors that restrict colonization of the bladder by the *cya* and *crp* UPEC mutants (197).

Microscopy has played a particularly important role in defining pathogenic processes in murine UTI models, revealing how UPEC strains interact with host tissues and bringing inflammatory responses into better focus. In one intriguing example, researchers surgically exposed kidneys in anesthetized rats and imaged living, infected tissue by using multiphoton microscopy following direct infusion of fluorescently labeled UPEC (71, 202). This work revealed an unexpected synergism between type 1 pili and other adhesive organelles known as P pili in promoting UPEC coloniza-

tion and biofilm formation within the proximal tubules of the kidneys.

Rodents, and mice in particular, have also been used to model systemic infections, mimicking episodes of bacteremia and urosepsis that can arise in human patients as serious complications of UTI (203–208). In these models, UPEC is inoculated directly into the bloodstream or delivered systemically via a subcutaneous or intraperitoneal injection. These approaches sidestep more natural routes in which UPEC disseminates systemically from sites within the urinary tract but have nonetheless provided valuable insight into the pathogenesis of urosepsis and related pathologies.

Murine models of UTI and associated infections have tremendous practical utility, being the major *in vivo* platform for addressing the efficacy and safety of new antibacterial therapeutics and vaccine candidates prior to testing in primates or humans. However, it should be emphasized that not all data obtained from murine studies are directly relevant to human UTIs. For example, by sensing bacterial products like flagellin, Toll-like receptor 11 (TLR11) stimulates host inflammatory responses, resulting in a modest but significant reduction in bacterial colonization of the kidneys in the mouse UTI model system (209, 210). However, in humans, the gene encoding TLR11 is not expressed due to the presence of a premature stop codon within an early exon and is therefore likely inconsequential as a defense against UTIs in human patients. Nonetheless, the identification of a functional role for TLR11 in mice highlights interesting questions concerning the evolution and operative redundancy of host defense factors. In the long run, these sorts of observations may also aid the development of new approaches to augment host resistance against UTIs and other infections.

Porcine

Like humans, many domesticated animals are susceptible to infection by UPEC and related pathogens (106, 211–215). Each year, thousands of pigs are diagnosed with pyelonephritis and culled in order to prevent widespread infection and contamination (216). Using porcine infection models, researchers have investigated the spectrum of host responses to pyelonephritis as well as the onset of renal scarring that can occur as a consequence of UTI (216–218). The latter problem is often associated with vesicoureteric reflux (VUR) and is especially problematic in human infants, but as children age beyond 4 years, the risk of renal scarring due to UTI drops precipitously (219, 220). In an *E. coli* infection model using both adult pigs and piglets with VUR, it was found that older animals were not any better protected from kidney scarring than piglets (218, 219). Extrapolating from these data, it was argued that aging does not intrinsically decrease the risk of kidney scarring but rather that children who are inherently more vulnerable to kidney scarring will already have developed scars before reaching the age of 4 years. In susceptible individuals, polymorphisms that affect signaling via TLRs or other host pathogen recognition receptors or that reduce the expression levels of cytokines like transforming growth factor β (TGF-β) may result in aberrant inflammatory responses that lead to increased bacterial growth and dissemination and decreased renal tubular function, ultimately causing host cell death and scarring (6, 44, 221). Bacterial toxins like alpha-hemolysin and adhesins like type 1, P, and F1C pili may initiate and/or exacerbate these pathological events. Although porcine models have proven utility in defining pathogenic processes during UTI, they currently lack the genetic tractability offered by some other animal model systems and are relatively expensive, precluding the use of large sample sizes. On the other hand, the ability of the porcine UTI model to recapitulate many important aspects of pyelonephritis in large mammals makes it a potentially highly relevant system for the assessment of novel therapeutics in preclinical trials prior to human studies.

Avian

UPEC strains are closely related to another group of bacteria known as avian-pathogenic *E. coli* (APEC), the causative agent of avian colibacillosis (222–225). This similarity suggests that analysis of APEC strains in their natural avian hosts might provide insight into UPEC-associated virulence mechanisms and vice versa. Work addressing this possibility has employed infection models in which UPEC or APEC strains are inoculated into the air sac of adult chickens, given via subcutaneous injection of young chicks, or injected into the allantoic cavity of fertilized chicken eggs (222, 226–228). In some studies, bacterial pathogenicity within the chicken model correlates well with virulence in the mouse urinary tract, highlighting the zoonotic potential of APEC strains (227–229). However, as a model for UTI, chickens and the embryonic chick model have some notable disadvantages. Chief among them is the fact that chicks lack a urinary bladder and likely present UPEC and related strains with stresses and opportunities distinct from those encountered within mammalian hosts. Despite these limitations, the low cost and ease of the embryonic chick model system provide an enticing alternative approach for screening UPEC-associated virulence determinants within a naturally susceptible vertebrate host. Furthermore, because UPEC may acquire many antibiotic resistance and virulence genes from APEC strains via horizontal gene transfer (227, 230), avian infection models might allow researchers to better discern selective pressures that affect the evolution of uropathogens.

Zebrafish

Recently, the teleost fish *Danio rerio*, commonly known as zebrafish, was developed as an additional host model system for the analysis of infections caused by UPEC and related pathogens (231, 232). To initiate infection, zebrafish embryos at 48 h postfertilization (hpf) are injected with UPEC at one of two sites: a fluid-filled sac surrounding the heart, referred to as the pericardial cavity, or the blood, via the circulation valley. Inoculation of the pericardial cavity limits bacterial dissemination within the embryo and serves as a model for localized infection. The introduction of UPEC into the circulation valley leads to the dispersal of bacteria throughout the bloodstream of the embryo, mimicking a systemic, sepsis-like infection. Each route of inoculation is thought to present UPEC with distinct challenges in terms of the types and levels of antimicrobial factors, phagocyte numbers, and nutrient availability (204, 231). At 48 hpf, zebrafish have only innate immune defenses, including antimicrobial peptides, complement, cytokines, TLRs and other microbial pattern recognition receptors, neutrophils, and macrophages (233–237). Homologous host defenses are central to the ability of both mice and humans to effectively resist UPEC (43, 44).

Relative to many other available model systems, zebrafish embryos are inexpensive and readily amenable to genetic manipulation and to medium- to high-throughput screens. Consequently, the growth and survival of large numbers of wild-type and mutant UPEC strains can be rapidly screened within the zebrafish host,

and pathogen effects on zebrafish morbidity and mortality can be readily assessed. Within a single day, hundreds of fish can be injected, with minimal costs, taking up only a rack or two within an incubator. Zebrafish embryos are also transparent, allowing easy, real-time observation of the infection process, including visualization of fluorescent gene expression reporters as well as fluorescently tagged bacteria, proteins, and host cell lineages (207, 231). Heart rate and blood flow are also easily monitored in the transparent embryos, along with bacterial dissemination and the development of necrotic lesions or other anomalies in host tissues. The effects of specific host genes on the infection process can be examined by using multiple approaches common to many model systems, including microarray analysis and RNA-seq. In addition, zebrafish genes can be silenced by using antisense RNA molecules known as morpholino oligomers (231) or specifically knocked out by using TALEN- or emerging CRISPR-Cas9-mediated genomic modification approaches (238, 239). Transgenic zebrafish that express foreign genes under the control of constitutive or cell-specific promoters can also be generated by using relatively straightforward approaches (240, 241).

In some instances, the zebrafish model is able to discern pathogenic phenotypes that are sometimes less clear-cut in mice or other host model systems. For example, the pore-forming toxin alpha-hemolysin (HlyA), which is produced by about 50% of all UPEC isolates, can promote the lysis of red blood cells, potentially freeing much-needed iron for use by the pathogens (98). Alternatively, at sublytic concentrations, HlyA can inhibit host cell inflammatory and survival pathways and indirectly stimulate the degradation of host cytoskeletal components (98, 174, 175, 242). In wild-type zebrafish, the survival of a reference UPEC isolate known as UTI89 is entirely dependent upon the expression of HlyA (231). However, in fish that lack phagocytes due to morpholino-mediated knockdown of the host transcription factor PU.1, UTI89 does not require HlyA for survival or virulence. This finding, along with microscopic imaging of infected zebrafish, indicates that a primary function of HlyA is to incapacitate phagocytes, with effects on red blood cell lysis and iron acquisition being secondary. Interestingly, HlyA is not required by all UPEC isolates for survival in zebrafish, suggesting that these pathogens can utilize various mechanisms to counter host phagocytes.

Despite the many benefits afforded by the zebrafish infection model, there are some caveats to this host system. Foremost is the lack of a mammal-like urinary tract in zebrafish, which prevents specific analysis of UTI pathogenesis. In addition, zebrafish are typically maintained at 28.5°C, notably lower than the temperature in most mammalian hosts. Nonetheless, zebrafish have proven to be remarkably useful for delineating UPEC-associated fitness and virulence factors that are relevant to the infection process in mammals (203, 204, 207, 231, 243). The discovery and functional analysis of both bacterial and host factors of importance to UPEC pathogenesis will likely advance in strides as imaging and genetic approaches in the zebrafish model continue to be developed and improved.

Nematodes

The nematode *Caenorhabditis elegans*, which probably comes into contact with *E. coli* within soil environments, is an attractively facile tool for exploring the virulence potential of UPEC and related *E. coli* isolates (232, 244, 245). These transparent worms consume bacteria as their main food source, which makes bacterial infections of these animals a trivial task. Once placed onto medium containing a pathogenic bacterial strain, the nematodes will graze and consequently become infected. By monitoring the death rate of worms infected with wild-type UPEC strains or isogenic mutants, the effects of defined loci on UPEC virulence can be ascertained. In addition, the roles of specific host genes during infection can be easily assessed by using RNA interference (RNAi), a process initiated by feeding the worms nonpathogenic bacteria that express double-stranded RNA molecules with homology to host messages of interest (246, 247). Nematodes encode a number of innate host defenses and signaling pathways with analogues in mammals, but the worms lack a true vertebrate-like immune system and have no urinary tract (248). As with zebrafish and embryonic chick models, limitations associated with the use of *C. elegans* as a surrogate host for UPEC may be countered by the low cost, genetic tractability, and amenability to high-throughput analysis.

CONCLUDING REMARKS

The virulence traits associated with UPEC are likely adaptations of genes that evolved long before modern humans arrived on the planet. For example, genetic factors that enabled ancestral *E. coli* strains to better avoid killing by predatory amebas in the environment may have later been coopted by UPEC to evade professional phagocytes within human hosts (249). Such conservation of function means that information gleaned by analysis of UPEC-associated fitness and virulence factors within one model system may be relevant in other environments. Consequently, the use of surrogate hosts like *C. elegans* and zebrafish, or even larvae of the wax moth *Galleria mellonella* (250–252), is not so tangential to UTI research and has the potential to drive important discoveries in human patients. Findings made by using animal model systems can be complemented and extended in broth culture or on agar plates using genetic and biochemical assays that are designed to examine how UPEC mutants and wild-type strains handle specific stresses such as reactive oxygen species or serum components like complement (197, 243). Mathematical and computer modeling of infectious processes and gene regulation in UPEC provides additional insight into the pathogenesis of UTIs and can suggest new hypotheses to be tested *in vivo* (46, 253, 254). In total, these various experimental approaches have helped define the molecular pathology of UTIs and the virulence potentials of UPEC isolates, highlighting numerous processes and gene products that may one day be of significant value as therapeutic targets.

ACKNOWLEDGMENTS

Research in the Mulvey laboratory is supported by grant AI095647 from the National Institutes of Health. A.E.B. was supported by NIH microbial pathogenesis training grant T32 AI055434. J.P.N. and T.J.W. were supported by NIH genetics training grant T32 GM007464.

We declare no conflict of interest.

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APPENDIX C

THE CPX STRESS RESPONSE SYSTEM POTENTIATES THE FITNESS OF UROPATHOGENIC *ESCHERICHIA COLI*

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Debnath I, Norton JP, Barber AE, Ott EM, Dhakal BK, Kulesus RR, Mulvey MA. The Cpx Stress Response System Potentiates the Fitness and Virulence of Uropathogenic *Escherichia coli*. *Infection & Immunity*. 2013;81(5):1450-9.
doi: 10.1128/IAI.01213-12.

IAI Journals.ASM.org

The Cpx Stress Response System Potentiates the Fitness and Virulence of Uropathogenic *Escherichia coli*

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Strains of uropathogenic *Escherichia coli* (UPEC) are the primary cause of urinary tract infections, representing one of the most widespread and successful groups of pathogens on the planet. To colonize and persist within the urinary tract, UPEC must be able to sense and respond appropriately to environmental stresses, many of which can compromise the bacterial envelope. The Cpx two-component envelope stress response system is comprised of the inner membrane histidine kinase CpxA, the cytosolic response regulator CpxR, and the periplasmic auxiliary factor CpxP. Here, by using deletion mutants along with mouse and zebrafish infection models, we show that the Cpx system is critical to the fitness and virulence of two reference UPEC strains, the cystitis isolate UTI89 and the urosepsis isolate CFT073. Specifically, deletion of the *cpxRA* operon impaired the ability of UTI89 to colonize the murine bladder and greatly reduced the virulence of CFT073 during both systemic and localized infections within zebrafish embryos. These defects coincided with diminished host cell invasion by UTI89 and increased sensitivity of both strains to complement-mediated killing and the aminoglycoside antibiotic amikacin. Results obtained with the *cpxP* deletion mutants were more complicated, indicating variable strain-dependent and niche-specific requirements for this well-conserved auxiliary factor.

Urinary tract infections (UTIs) afflict a large proportion of the human population, representing an enormous health and financial burden worldwide (1). Most UTIs are caused by a genetically diverse group of bacteria known as uropathogenic *Escherichia coli* (UPEC). These pathogens can survive and grow within urine and the lumen of the bladder, but many can also bind and invade uroepithelial cells (2–4). Within the bladder, entry into uroepithelial cells can promote UPEC survival and persistence, rendering the pathogens protected from a variety of stresses and commonly used antibiotics (3, 5, 6). Prior to introduction into the urinary tract, UPEC likely first colonizes the host nasopharynx and gastrointestinal tract, where it does not appear to elicit any overt pathology (7–9). Within these varied host environments, and while in transit between hosts, UPEC will encounter an assorted array of stresses, including reactive nitrogen and oxygen species, nutrient limitation, shearing forces, professional phagocytes, complement and other antimicrobial compounds, competition with other microbes and, potentially, antibiotics (10–15). The ability to deal with these stresses is of paramount importance to the success of UPEC as a pathogen.

The envelope of Gram-negative bacteria interfaces with the extracellular environment, functioning as both a sensor of external conditions and as a selectively permeable physical barrier. Envelope stress response pathways are likely critical to the ability of UPEC to detect and respond to potentially fatal environmental insults during the course of infection. UPEC, as well as other *E. coli* strains, encode a number of envelope stress response systems, including sigma E (σ^E), Rcs, Psp, and the BaeSR and CpxRA two-component systems (16–19). The Cpx system is comprised of the inner membrane histidine kinase CpxA and the cytoplasmic response regulator CpxR (20–22). Autophosphorylation of CpxA in response to envelope stress results in the phosphorylation of CpxR, which then functions as a transcriptional regulator. CpxR controls the expression of protein folding and degrading factors involved in relieving envelope stress and can also regulate biofilm formation (23–26), bacterial adherence (23, 27, 28), motility and chemotaxis (29, 30), type III and type IV secretion systems (31–35) and, possibly, the synthesis of bacterial toxins (27, 36, 37). Studies using *E. coli* K-12 strains like MG1655 and MC4100 have indicated that CpxR may regulate the expression of well over 100 genes (38, 39).

In *E. coli* and other microbes, the Cpx system is subject to negative feedback through CpxP, a small CpxR-regulated periplasmic protein that can bind the sensor kinase CpxA, keeping it in an inactive state (40, 41). CpxR binding sites are situated upstream of the *cpxP* gene within a conserved 146-bp region that separates *cpxP* from the *cpxRA* operon. CpxP is the most highly inducible member of the Cpx regulon so far identified, and it has elevated expression in response to both envelope stress and entry into stationary-phase growth (40, 42). In addition to its role as a negative regulator of CpxA, CpxP also functions as an adaptor protein, interacting with subsets of misfolded periplasmic proteins and delivering them to the protease DegP for degradation (43, 44). In this process, CpxP is degraded along with its misfolded substrate, suggesting a mechanism by which bacteria can post-translationally modulate CpxP levels. By varying the amounts of CpxP within the periplasm, bacteria may be able to fine-tune the Cpx stress response, limiting inappropriate activation of CpxA in the absence of envelope stress and permitting rapid shutoff of the system once the stress is under control (20, 45).

The Cpx system appears to have a key role in regulating the virulence potential of a number of pathogens (17), including *Sal-*

Received 1 November 2012 **Returned for modification** 11 December 2012
Accepted 12 February 2013
Published ahead of print 19 February 2013
Editor: S. M. Payne
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doi:10.1128/IAI.01213-12

TABLE 1 Bacterial strains and plasmids

Strain or plasmid	Description	Source or reference(s)
Wild-type strains		
UTI89	UPEC, cystitis isolate (O18:K1:H7)	6, 95
CFT073	Urosepsis isolate (O6:K2:H1)	96
Recombinant strains		
UTI89Δ*cpxP*	UTI89 Δ*cpxP*::clm^r	This study
UTI89Δ*cpxRA*	UTI89 Δ*cpxRA*::clm^r	This study
UTI89Δ*fieF*	UTI89 Δ*fieF*::clm^r	This study
CFT073Δ*cpxP*	CFT073 Δ*cpxP*::clm^r	This study
CFT073Δ*cpxRA*	CFT073 Δ*cpxRA*::clm^r	This study
CFT073Δ*fieF*	CFT073 Δ*fieF*::clm^r	This study
Plasmids		
pKM208	IPTG-inducible Red recombinase expression plasmid, Amp^r	60
pGEN-MCS	High-retention plasmid containing empty multiple-cloning site, Amp^r	61
pJLJ41p	*cpxP* sequence with native promoter from UTI89 cloned into pGEN-MCS; Amp^r	This study
pJLJ42	*cpxRA* sequence with native promoter from UTI89 cloned into pGEN-MCS; Amp^r	This study
pNLP10-*lux*	Low-copy-number cloning vector with promoterless *luxCDABE* operon; Kan^r	39
pJW1-c*pxP-lux*	pNLP10 with P*cpxP*::*luxCDABE*, Kan^r	39

monella spp. (46, 47), *Legionella pneumophila* (31, 48), *Shigella* spp. (33–35), enteropathogenic *E. coli* (32, 49, 50), *Actinobacillus suis* (51), *Haemophilus ducreyi* (52, 53), *Xenorhabdus nematophila* (37, 54), and *Yersinia pseudotuberculosis* (55–57). However, direct evidence that the Cpx system can affect pathogen fitness and virulence *in vivo* within an animal host is limited to only a few studies (47, 50, 53, 54). In UPEC, the Cpx system has been examined primarily with respect to its ability to modulate the expression of P pili, filamentous adhesive organelles that can promote bacterial interactions with host kidney cells (27, 28, 58). Here, by using isogenic deletion mutants, we assessed how components of the Cpx stress response system affect the fitness and virulence of two reference UPEC isolates. Employing *in vitro* assays coupled with *in vivo* mouse and zebrafish infection models, we demonstrate that *cpxP* and *cpxRA* can have profound and sometimes divergent effects on the pathogenic potential of UPEC.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. Targeted gene knockouts were created in the human cystitis isolate UTI89 and the urosepsis isolate CFT073 by using lambda Red-based homologous recombination as previously described (59, 60). Briefly, the chloramphenicol resistance cassette (clm^r) was amplified from the template plasmid pKD3 with flanking 40-bp overhangs specific for the target *cpxP* or *cpxRA* loci. PCR products were electroporated into UTI89 and CFT073 carrying the plasmid pKM208, which encodes an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible lambda Red recombinase. The *yiiP* (*fieF*) gene was knocked out by using a similar approach. Knockout strains were selected on Luria-Bertani (LB) agar plates containing chloramphenicol (20 μg/ml) and verified by PCR using the primers listed in Table 2.

Expression constructs were made using the low-copy-number plasmid pGEN-MCS and standard molecular biology techniques (61). The *cpxP* gene and the *cpxRA* operon were cloned by PCR from the UTI89 chromosome. The primers used (Table 2) to amplify each locus were designed to include 250 bp of upstream and 100 bp of downstream sequences, along with terminal PstI and SalI restriction sites. PCR products were cut using PstI and SalI and ligated into pGEN-MCS to create the CpxP and CpxRA expression constructs pJLJ41p and pJLJ42, respectively.

TABLE 2 Primers used in this study[a]

Primer	Sequence (5′–3′)
cpxP KO	
Forward	ATGCGCATAGTTACCGCTGCCGTCATGGCCT CAACGCTGGGTGTAGGCTGGAGCTGCTTC
Reverse	CTACTGGGAACGTGAGTTGCTACTACTCAATA GCTTCAACCATATGAATATCCTCCTTAG
cpxP confirmation	
Forward	CTATCGTTGAATCGCGACAG
Reverse	GGATGGTGTCTATGGCAAGG
cpxRA KO	
Forward	ATGAATAAAATCCTGTTAGTTGATGATGACC GAGAGCTGGTGTAGGCTGGAGCTGCTTC
Reverse	TTAACTCCGCTTATACAGCGGCAACCAAATC ACCAGCCGTCATATGAATATCCTCCTTAG
cpxRA confirmation	
Forward	ACTGCCAGCGTTGAGGCCATGA
Reverse	GAGTGTAGGCCTGATAAGACGCTATCAGC
fieF KO	
Forward	ATGAATCAATCTTATGGACGGCTGGTCAGTC GGGCGGCTGTGTAGGCTGGAGCTGCTTCG
Reverse	TTATGAAAGCATAGACCGTTTACCCTCCCTG GGTACGACGCATATGAATATCCTCCTTAG
fieF confirmation	
Forward	CCTTGCCATAGACACCATC
Reverse	TCAGGTCAGGCCAAATGG
pJLJ41p	
Forward (PstI)	AATCCTGCAGATTGTTTAAATACCTCCGAGGC
Reverse (SalI)	TAGAGTCGACTACCAGCGCGGCGAGAATAC
pJLJ42	
Forward (PstI)	TGCTCTGCAGTCATTTGCTCCCAAAATCTTTCT
Reverse (SalI)	GCTAGTCGACAGCGGCAAGATCGAAGATTTTT

[a] Added restriction sites underlined. KO, knockout.

Growth curves. Bacteria were grown from frozen stocks at 37°C with shaking overnight in 5 ml of LB broth or modified M9 minimal medium (6 g/liter Na_2HPO_4, 3 g/liter KH_2PO_4, 1 g/liter NH_4Cl, 0.5 g/liter NaCl, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 0.1% glucose, 0.0025% nicotinic acid, 16.5 μg/ml thiamine, and 0.2% casein amino acids). Cultures were then diluted 1:100 into the indicated medium, and the growth of quadruplicate 200-μl samples in shaking 100-well honeycomb plates at 37°C was assessed using a Bioscreen C instrument (Growth Curves USA). For competition assays, wild-type and mutant strains diluted 1:200 were mixed at a 1-to-1 ratio in 5 ml modified M9 medium and grown with shaking at 37°C. After 2, 4, and 6 h of growth, titers of the mutant and wild-type strains were determined by plating serial dilutions on LB agar with or without chloramphenicol (to distinguish wild-type and mutant strains). Competitive indices were calculated as follows: $\log_{10}[(\text{mutant}_{\text{output}}/\text{wild-type}_{\text{output}})/(\text{mutant}_{\text{input}}/\text{wild-type}_{\text{input}})]$. Media and other reagents used in these assays were purchased from Sigma-Aldrich.

Amikacin susceptibility assays. Bacteria were grown from frozen stocks with shaking at 37°C in 5 ml modified M9 medium with or without 100 μg/ml ampicillin (used to maintain plasmids). Overnight cultures were diluted 1:100 into 1 ml modified M9 medium containing amikacin at concentrations ranging from 1 to 40 μg/ml. Each culture was then grown with shaking at 37°C for 24 h. The MIC of each strain was determined as the lowest concentration of amikacin needed to prevent growth.

***cpxP* promoter activity assays.** Bacteria carrying pNLP10-*lux* or pJW1-*cpxP-lux* were grown from frozen stocks at 37°C with shaking overnight in 5 ml modified M9 medium containing 50 μg/ml kanamycin (39). Overnight cultures were diluted 1:100 into 5 ml modified M9 medium containing 50 μg/ml kanamycin and incubated with shaking at 37°C for 4 h to reach stationary phase (optical density at 600 nm, ≈1.0). Triplicate 100-μl aliquots of each sample were then transferred into a 96-well white, opaque-walled polystyrene microplate (Dynex Technologies), and luminescence was measured immediately with a Synergy HT multidetection microplate reader (BioTek Instruments, Inc.).

Hemagglutination assays. Hemagglutination titers were determined using guinea pig red blood cells (Colorado Serum Company) as described previously (62). Bacteria used in these assays were grown statically from frozen stocks in 20 ml modified M9 medium or LB broth for 48 h at 37°C.

Mouse UTI model. Seven- to 8-week-old female CBA/J mice (Jackson Laboratory) were used, following IACUC-approved protocols as previously described (5, 63, 64). Wild-type and mutant bacterial strains were grown from frozen stocks in 20 ml static modified M9 medium for 24 h at 37°C. Prior to inoculation, bacteria were pelleted by centrifugation for 10 min at 8,000 × *g* and then resuspended in phosphate-buffered saline (PBS). Mice were anesthetized by using isoflurane inhalation and carefully inoculated by transurethral catheterization with 50 μl of a bacterial suspension containing 1×10^7 CFU. For competitive assays, wild-type UTI89 was mixed 1:1 with either the Δ*cpxP* or Δ*cpxRA* mutant prior to inoculation. For noncompetitive assays, each strain was inoculated separately. Bladders were harvested aseptically at 3 days postinoculation, weighed, and homogenized in sterile PBS containing 0.02% Triton X-100. Bacterial titers present in the input pools and in the bladder homogenates were determined by plating serial dilutions on LB agar plates. For competitive assays, LB agar plates with or without chloramphenicol (20 μg/ml) were used to distinguish the wild-type and mutant strains. Competitive indices were calculated as follows: $\log_{10}[(\text{mutant CFU inoculated}/\text{wild-type CFU inoculated})/(\text{mutant CFU recovered}/\text{wild-type CFU recovered})]$; based on this equation, values of less than 0 indicated that the wild-type strain outcompeted the mutant. Experiments were repeated two to three times, and combined data are shown.

Zebrafish infections. Zebrafish used in this study were handled in accordance with IACUC-approved protocols and following standard procedures (www.zfin.org), as previously described (65). *AB zebrafish embryos were collected from mixed egg clutches in a breeding colony that was maintained on a 14-h light/10-h dark cycle. Embryos were grown at 28.5°C in E3 medium (5 mM NaCl, 0.27 mM KCl, 0.4 mM $CaCl_2$, 0.16 mM $MgSO_4$) containing 0.000016% methylene blue as an antifungal agent. At 48 h postfertilization (hpf), embryos were manually dechorianated, briefly anesthetized with 0.77 mM ethyl 3-aminobenzoate methanesulfonate salt (Tricaine; Sigma-Aldrich), and embedded in low-melting-point agarose (Mo Bio Laboratories) without Tricaine. Agarose-embedded embryos were then transferred to E3 medium lacking methylene blue and infected individually with wild-type CFT073 or the *cpx* mutants. Bacteria were grown from frozen stocks, held static in 12 ml modified M9 medium at 37°C for 24 h. One milliliter from each culture was pelleted, washed once with 1 ml PBS, and resuspended in PBS prior to inoculation into either the pericardial cavity or circulation valley by using an Olympus SZ61 or SZX10 stereomicroscope together with a YOU-1 micromanipulator (Narishige), a Narishige IM-200 microinjector, and a JUN-AIR model 3 compressor setup. For each bacterial strain, 500 to 1,000 CFU suspended in 1 nl PBS was injected per fish. Inoculation titers were determined by adding 10 drops (1 nl each) to 1 ml 0.7% NaCl, which was then serially diluted and plated on LB agar plates. Following injection, embryos were carefully removed from the agar, placed individually into wells of a 48-well plate (Nunc) containing E3 medium, and incubated at 28.5°C. Fish viability was assessed at regular intervals for 72 h following injection by monitoring heart beats and blood flow.

Bacterial host cell association and invasion assays. Host cell association and gentamicin protection-based invasion assays were performed as previously described (66, 67). Strains used in these assays were grown at 37°C for 48 h in static LB broth to induce expression of type 1 pili. Human bladder epithelial cells, designated 5637 (HTB-9; ATCC), were grown at 37°C in 5% CO_2 using RPMI 1640 medium (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (HyClone). Bladder cells were infected with a multiplicity of infection of ~15 bacteria per host cell.

Serum resistance assays. Frozen aliquots of pooled human sera, taken from 7 healthy volunteers by using standard protocols approved by the University of Utah Institutional Review Board, were provided by Andrew Weyrich. Care was taken to not freeze and thaw samples multiple times. Bacteria from overnight cultures grown with shaking at 37°C in modified M9 medium were pelleted by spinning at 8,000 × *g* for 5 min, washed twice, and resuspended in PBS to obtain $\sim 1 \times 10^6$ CFU/ml. About 5×10^4 CFU of each bacterial strain was mixed individually with modified M9 medium containing 20% serum, and 200-μl aliquots of each suspension were immediately placed in a 96-well microtiter plate and incubated with gentle shaking for 2.5 h at 37°C. Plates were then placed on ice, and surviving bacteria were enumerated by plating serial dilutions on LB agar. Results were normalized to input titers. Heat-inactivated serum (treated at 55°C for 30 min) was used as a negative control.

Statistical analysis. The Mann-Whitney U test, Wilcoxon matched pair test, log-rank (Mantel-Cox) test, and Student's *t* test were performed using Prism 5.01 software (GraphPad Software). *P* values of less than 0.05 were defined as significant.

RESULTS

The Cpx system modulates UPEC resistance to amikacin. By using lambda Red-mediated linear recombination, the *cpxRA* operon and the *cpxP* gene were individually deleted from two reference UPEC strains, the cystitis isolate UTI89 and the urosepsis isolate CFT073. As the first step in our efforts to phenotypically characterize these mutants, we assessed their sensitivities to the aminoglycoside antibiotic amikacin. Previous studies showed that laboratory K-12 *E. coli* strains lacking *cpxA* or *cpxRA* have increased sensitivity to amikacin, whereas induction of the Cpx pathway or the expression of constitutively active *cpxA* mutants (*cpxA**) provides strains with improved resistance to amikacin (68–70). Resistance has been attributed to the ability of the Cpx system to activate transcription of drug exporters as well as factors that help alleviate the stress of mistranslated proteins that may

TABLE 3 Amikacin MIC assay results[a]

Strain	MIC (μg/ml)
UTI89	18
UTI89Δ*cpxRA*	6
UTI89Δ*cpxP*	28
UTI89(pGEN-MCS)	16
UTI89Δ*cpxRA*(pJLJ42)	30
UTI89Δ*cpxP*(pJLJ41p)	18
CFT073	20
CFT073Δ*cpxRA*	6
CFT073Δ*cpxP*	30
CFT073(pGEN-MCS)	20
CFT073Δ*cpxRA*(pJLJ42)	16
CFT073Δ*cpxP*(pJLJ41p)	20

[a] The assay was repeated three times and the same results were obtained.

accumulate within the bacterial envelope due to amikacin effects on ribosome activity (25, 70, 71).

In agreement with results obtained using K-12 strains (68, 69), we observed that both UTI89Δ*cpxRA* and CFT073Δ*cpxRA* were highly sensitive to amikacin relative to their wild-type counterparts, as determined by MIC assays (Table 3). Plasmid pJLJ42, which carries the *cpxRA* operon under the control of its native promoter, complemented both Δ*cpxRA* mutants, whereas the empty vector pGEN-MCS had no effect (Table 3 and unpublished observations). Deletion of *cpxP*, which leaves CpxA less repressed (40, 45), rendered UTI89 and CFT073 notably more resistant to amikacin (Table 3). Expression of recombinant *cpxP* using plasmid pJLJ41 restored the resistance of the Δ*cpxP* mutants to wild-type levels. Of note, deletion of the gene *yiiP* (*fieF*) located immediately downstream of *cpxP* did not affect the sensitivity of either UTI89 or CFT073 to amikacin (unpublished observations).

These results indicate that the Cpx response in the UPEC isolates operates, not unexpectedly, similarly to the Cpx response in K-12 strains. To further address this point, we utilized a low-copy-number reporter construct containing the *cpxP* promoter fused to a promoterless *luxCDABE* operon (39). In both CFT073 and UTI89, the deletion of *cpxRA* ablated expression of the *cpxP* reporter in early-stationary-phase cultures, whereas deletion of *cpxP* greatly enhanced expression (Fig. 1). These data parallel those reported for similar assays carried out with K-12 strains, supporting models in which CpxP functions in part as a negative regulator of Cpx activation (40, 45).

The Cpx system provides UPEC with a fitness advantage within the bladder. To address whether or not the Cpx system can affect the fitness of UPEC within the urinary tract, we utilized a well-established UTI model system, focusing on bladder colonization by the cystitis isolate UTI89. Adult female CBA/J mice were inoculated via transurethral catheterization with wild-type UTI89, UTI89Δ*cpxRA* or UTI89Δ*cpxP*, and 3 days later bacterial titers within the bladder were determined. In noncompetitive assays, in which equal numbers of the wild-type and mutant strains were inoculated separately into different mice, the Δ*cpxRA* mutant was recovered in significantly lower numbers than wild-type UTI89 (Fig. 2A). In contrast, no significant difference was observed between wild-type UTI89 and the Δ*cpxP* mutant. Similar results were obtained in competitive assays, in which the wild-type strain was mixed 1:1 with each mutant strain prior to inoculation (Fig. 2B and C). These results indicated that *cpxRA*, but not *cpxP*, is required by UTI89 to effectively colonize the bladder.

During the course of a UTI, UPEC comes across a variety of environmental stresses that can potentially limit its survival and growth within the host (10–15). These stresses include reactive nitrogen and oxygen radicals and numerous membrane-damaging substances. In LB broth and modified M9 medium, the *cpxRA* and *cpxP* deletion mutants grew normally, whether on their own in monoculture or in direct competition with the wild-type strains (Fig. 3). Likewise, no defects were observed with the Δ*cpxRA* or Δ*cpxP* mutants when challenged *in vitro* with nitrosative stress (1 mM acidified sodium nitrite), oxidative stress (0.5 or 1 M methyl viologen), or envelope stress generated by addition of 0.1% sodium dodecyl sulfate (unpublished observations). These findings indicate that deletion of *cpxRA* or *cpxP* does not alter the ability of UPEC to handle generalized stresses.

Divergent effects of the Cpx system on host cell invasion by UPEC. Effective colonization of the bladder by UPEC generally requires the expression of functional type 1 pili (63, 72–75). These filamentous adhesive organelles mediate bacterial attachment to and invasion of bladder epithelial cells, promoting the establish-

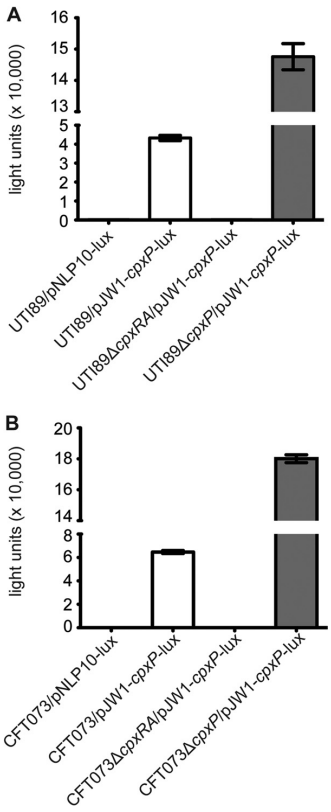

FIG 1 Deletion of *cpxP* enhances Cpx activation in both CFT073 and UTI89. Graphs indicate expression levels (± standard deviations) of the *luxCDABE* operon driven by the *cpxP* promoter in wild-type UTI89 (A) and wild-type CFT073 (B) and their mutant derivatives, following growth to early stationary phase in modified M9 medium. The pNLP1-*lux* plasmid carries a promoterless *luxCDABE* operon. Each graph shows the means ± standard errors of the means of three independent experiments performed in triplicate.

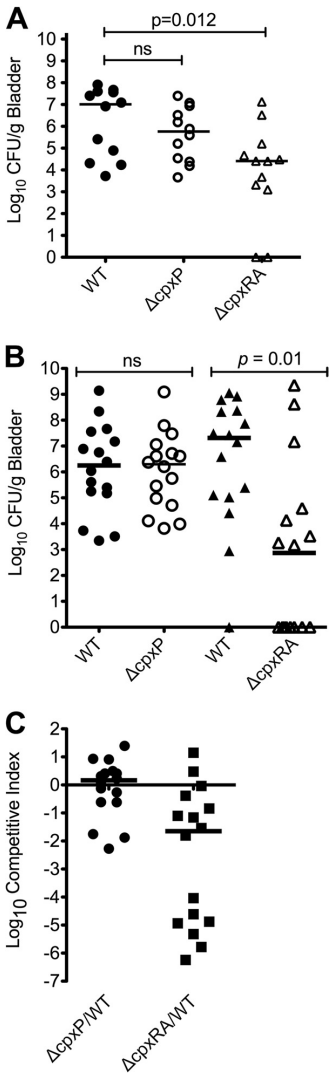

FIG 2 The Cpx system promotes UPEC fitness within the bladder. Adult female CBA/J mice were infected via catheterization with wild-type UTI89, UTI89Δ*cpxP*, or UTI89Δ*cpxRA* in noncompetitive (A) and competitive (B and C) assays. Graphs show bacterial titers present in the bladder at 3 days postinoculation. Bars denote median values for each group ($n \geq 12$ mice). The data in panel B are graphed in panel C as competitive indices. *P* values were determined using the Mann-Whitney U test (A) or Wilcoxon-matched paired signed rank test (B). ns, no significant difference.

ment and persistence of UPEC within the urinary tract (5, 6, 63, 76). In yeast agglutination assays, as well as hemagglutination assays performed using guinea pig red blood cells, we observed no overt differences in the expression of type 1 pili by the Δ*cpxRA* or Δ*cpxP* mutants relative to the wild-type UPEC isolates (unpublished observations). However, UTI89Δ*cpxRA* did show a slight, but significant (~20%), decrease in its ability to adhere to human bladder epithelial cells in culture (Fig. 4A). This reduction in adherence corresponded with a similar (~30%) decrease in host cell invasion by UTI89Δ*cpxRA*, as determined in gentamicin protection assays (Fig. 4B). These modest defects in host cell adherence and invasion could be rescued by complementation of the Δ*cpxRA* mutant with pJLJ42. In contrast to UTI89Δ*cpxRA*, the Δ*cpxP* mutant had no defect in its ability to bind bladder epithelial cells (Fig. 4A), but it was able to invade the host cells at a much higher frequency than either wild-type UTI89 or the Δ*cpxRA* mutant (Fig. 4B). Complementation of UTI89Δ*cpxP* with pJLJ42 reduced the invasion frequencies of this mutant to wild-type levels. Importantly, wild-type UTI89 and the Δ*cpxRA* and Δ*cpxP* mutants grew similarly in the cell culture medium, and all three strains were equally susceptible to killing by gentamicin at the concentration (100 μg/ml) used in these invasion assays.

Cpx components promote UPEC virulence in zebrafish. To assess effects of the Cpx system on UPEC virulence, and not fitness *per se*, we next focused on CFT073 in a zebrafish infection model that was recently developed in our laboratory (65). In this model system, bacteria are microinjected into 48-hpf zebrafish embryos via either a fluid-filled sac surrounding the heart, known as the pericardial cavity (PC), or directly into the bloodstream through the circulation valley. UPEC does not usually spread from the PC, whereas the pathogens rapidly disseminate systemically following inoculation of the bloodstream. At 48 hpf, zebrafish are dependent upon innate host defenses that include phagocytes, antimicrobial peptides, and complement—the same sort of defenses that mammalian hosts employ against UPEC (77–81). The use of zebrafish has proven to be an effective way to identify and functionally define virulence factors of relevance to UPEC and related pathogens that can colonize an assorted array of hosts and host tissues (unpublished observations and references 65 and 82).

Relative to UTI89 and many other UPEC isolates, CFT073 is especially lethal to zebrafish embryos (65). Here, we compared the lethality of wild-type CFT073 to CFT073Δ*cpxRA* and CFT073Δ*cpxP* following inoculation of 500 to 1,000 CFU of each strain individually into the PC or blood. In this infection model, increased bacterial growth correlates with decreased host survival (65). Wild-type CFT073 killed most of the zebrafish embryos within 24 h, irrespective of the site of inoculation (Fig. 5). In comparison to the wild-type strain, the virulence of both the Δ*cpxRA* and Δ*cpxP* mutants was significantly decreased. Virulence defects associated with CFT073Δ*cpxRA* and CFT073Δ*cpxP* were particularly evident following inoculation of the blood (Fig. 5B), which in general appears to be a more challenging and stressful environment than the PC (65). Wild-type CFT073 and the Δ*cpxRA* and Δ*cpxP* mutants grew similarly in modified M9 minimal medium at 28.5°C, the temperature at which the zebrafish embryos are maintained. Plasmid pJLJ42 (*cpxRA*) and pJLJ41 (*cpxP*) rescued the virulence defects associated with CFT073Δ*cpxRA* and CFT073Δ*cpxP*, respectively, but the wild-type strain carrying empty vector was attenuated, complicating interpretation of our *in vivo* complementation assays (unpublished observations).

Strain-dependent effects of Cpx components on serum resistance. Urine, like serum, contains numerous antibacterial factors, including heat-labile components of the complement system that can mediate bacterial opsonization and the formation of membrane attack complexes (14, 83–85). By modulating the composition and resilience of the bacterial envelope, we hypothesized that

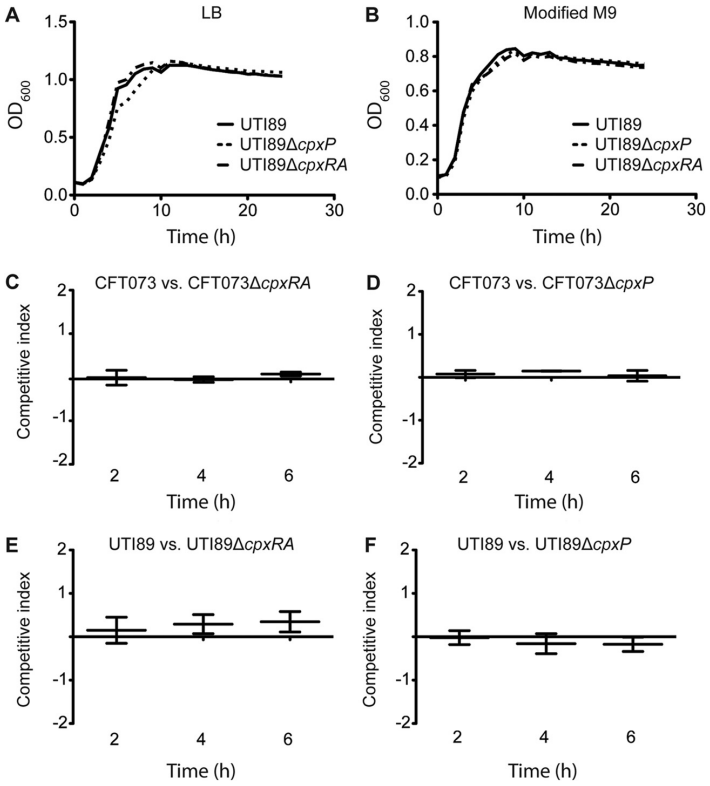

FIG 3 CFT073 and UTI89 mutants lacking either *cpxP* or *cpxRA* grow normally in LB broth and modified M9 medim. (A and B) Growth of wild-type UTI89 and associated Δ*cpxP* and Δ*cpxRA* mutants grown in LB broth (A) and modified M9 medium (B). Graphs are representative of at least three independent experiments performed in quadruplicate. (C to F) Results of competitive growth assays carried out in modified M9 medium with wild-type CFT073 and UTI89 versus isogenic Δ*cpxRA* or Δ*cpxP* mutants, as indicated. Data are presented as box-and-whiskers plots, with means ± the minimum and maximum values from three independent experiments.

the Cpx system can alter the sensitivity of UPEC to serum components. To examine this possibility, serum resistance assays were performed using wild-type UTI89 and CFT073 along with the Δ*cpxRA* and Δ*cpxP* mutants. In these assays, both UTI89Δ*cpxRA* and CFT073Δ*cpxRA* were significantly more sensitive to pooled human sera than their wild-type counterparts (Fig. 6A). UTI89Δ*cpxP* was likewise sensitive, whereas CFT073Δ*cpxP* showed no decrease in serum resistance relative to wild-type CFT073. Serum resistance defects associated with UTI89Δ*cpxRA*, CFT073Δ*cpxRA*, and UTI89Δ*cpxP* were rescued by plasmids carrying CpxRA (pJLJ42) or CpxP (pJLJ41), as appropriate (Fig. 6B). In assays that used heat-inactivated serum, which lacks functional complement, no differences were observed between the wild-type and mutant strains (Fig. 6C). Together, these data indicate that CpxRA, with strain-dependent input from CpxP, can enhance UPEC resistance to serum and, specifically, complement.

DISCUSSION

This study was aimed at delineating the impact of the Cpx envelope stress response system on the fitness and virulence of UPEC. Our results demonstrated that CpxRA and the auxiliary factor CpxP can affect the ability of UPEC to colonize distinct host environments. Employing a well-established mouse UTI model, we found that deletion of *cpxRA* limited the ability of the reference cystitis isolate UTI89 to effectively colonize the bladder, whereas deletion of *cpxP* had only modest effects. In laboratory K-12 *E. coli* strains, CpxP is not an essential regulator of the Cpx system, and instead it appears to modulate how quickly CpxA can be activated or inactivated in response to changing levels of envelope stress (20, 40, 43, 45). Within the bladder, the regulatory effects of CpxP are apparently dispensable to UTI89, at least at the 3-day time point that was analyzed. In contrast, deletion of either *cpxRA* or *cpxP* markedly attenuated the virulence of the urosepsis isolate CFT073 during both localized and systemic infections in zebrafish embryos. These data suggest that CpxP is differentially required by UPEC, depending upon strain background and the host environment. This idea was further supported by *in vitro* assays that showed that the resistance of UTI89 to complement-mediated killing was dependent upon both CpxRA and CpxP, while CFT073 required only CpxRA.

The Cpx system is intercalated within a complex web of signaling cascades and linked up with multiple biosynthetic and metabolic pathways (25, 38, 39, 41, 45, 86–88), making it difficult to

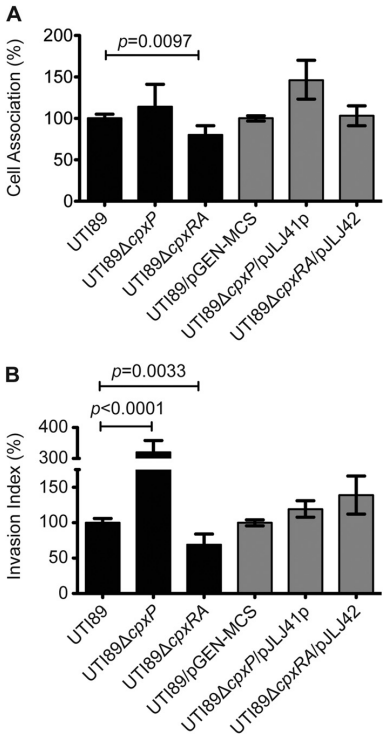

FIG 4 Cpx effects on bladder cell invasion by UTI89. Human bladder epithelial cells were infected with the indicated strains for 2 h, followed by an additional 2-h incubation in medium containing gentamicin (100 μg/ml). Graphs show the total cell-associated bacterial titers prior to addition of gentamicin (A) and for gentamicin-protected, intracellular bacteria (B). Data are expressed relative to wild-type UTI89 (black bars) or UTI89 carrying the control plasmid pGEN-MCS (gray bars) as the means ± standard errors of the means of at least three independent experiments performed in triplicate. The indicated *P* values were calculated using Student's *t* test.

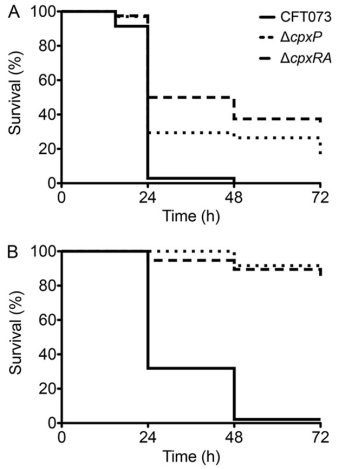

FIG 5 The Cpx system is required for full virulence of CFT073 in zebrafish embryos. The PC (A) or blood (B) of 48-hpf zebrafish embryos was inoculated with 500 to 1,000 CFU of wild-type CFT073, CFT073Δ*cpxP*, or CFT073Δ*cpxRA*, as indicated. Fish were scored for death at 0, 24, 48, and 72 h postinoculation. Data are expressed as the percent survival over time ($n \geq 17$ embryos). $P \leq 0.0008$ for the Δ*cpxP* and Δ*cpxRA* mutants versus control wild-type CFT073, as determined using Mantel-Cox log rank tests.

discern with clarity the specific mechanisms by which Cpx components moderate UPEC stress resistance and virulence phenotypes. It is clear, however, that basic regulation of the Cpx system in UPEC functions similarly to the Cpx system in nonpathogenic K-12 *E. coli* strains. In K-12 strains, CpxP regulates Cpx activation via a negative feedback loop (40), and this also appears to be the case in UPEC (Fig. 1). Furthermore, deletion of *cpxRA* rendered both UTI89 and CFT073 highly sensitive to the aminoglycoside amikacin (at 3 μg/ml), whereas deletion of *cpxP* increased amikacin resistance. These data are in agreement with work carried out in K-12 strains (68–70) and support the notion that activation of the Cpx system safeguards against aminoglycoside antibiotics. Protection is likely afforded by Cpx-mediated upregulation of proteases and other factors that alleviate envelope stress initiated by the mistranslation of inner membrane proteins in the presence of amikacin (70). Cpx activation may also heighten bacterial resistance to antibiotics via effects on the expression of drug transporters (25, 71).

The protective effects of Cpx activation are limited and will not shield against all concentrations and types of antibiotics, including the aminoglycoside gentamicin, used in our host cell invasion assays (70). This means that the slight but significant decrease in host cell invasion by UTI89Δ*cpxRA* and the elevated invasion frequencies seen with UTI89Δ*cpxP* are likely not attributable to Cpx-regulated effects on the susceptibility of UTI89 to gentamicin. Instead, the Cpx system may affect bacterial survival during or immediately after internalization or, alternatively, modulate the efficacy of the invasion process directly by affecting the surface characteristics of UPEC. The latter possibility is buoyed indirectly by observations showing that disruption of the Cpx system can alter bacterial interactions with hydrophobic abiotic surfaces (89). Of note, UPEC mutants lacking either *cpxRA* or *cpxP* were not obviously different from the wild-type strains with respect to motility, biofilm formation in microtiter plate assays, or the expression of curli or type 1 pili (unpublished observations). This indicates that many of the phenotypes commonly associated with UPEC virulence are unaffected by disruption of the Cpx system.

The reduced capacity of UTI89Δ*cpxRA* to bind and invade bladder epithelial cells may contribute to the inability of this mutant to effectively colonize the bladder. However, it is probable that additional CpxRA-regulated activities also play a role. These activities may include Cpx-mediated alterations of the bacterial envelope and peptidoglycan layer that enable bacteria to better deal with antimicrobial peptides and proteins like complement (90). The complement system can mediate bacterial opsonization and the formation of membrane attack complexes, thereby facilitating the clearance of bacteria during both localized and systemic infections (83). The strain-dependent requirement for CpxP in UPEC resistance to complement-mediated killing, as reported here (Fig. 6), highlights the individuality of UPEC isolates, which are often genetically diverse, while also raising questions regarding the functionality of highly conserved proteins like CpxP.

In addition to modulating the activity of CpxA, CpxP can func-

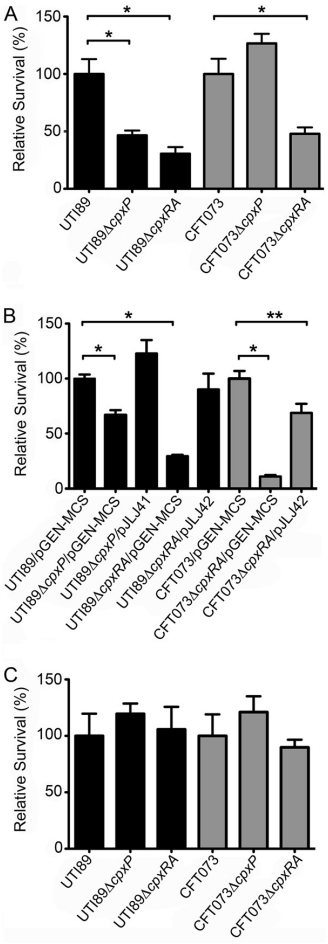

FIG 6 Cpx components have strain-dependent effects on serum resistance. About 5×10^4 CFU of wild-type UTI89 or CFT073 or their mutant derivatives were incubated at 37°C with gentle shaking in modified M9 medium containing 20% human serum (A) or 20% heat-inactivated serum (C). After 2.5 h, surviving bacteria were enumerated by plating serial dilutions. (B) Similar assays with 20% serum were performed using strains carrying plasmids pJLJ41 or pJLJ42 or the control empty vector, pGEN-MCS, as indicated. Data are presented relative to the wild-type strains as the means ± standard errors of the means of at least four independent experiments. In panel B, the control wild-type strains carried pGEN-MCS. *, $P < 0.007$; **, $P = 0.02$ (determined with Student's *t* test).

tion as a periplasmic chaperone and may act as a sensor for metal ions, like zinc and copper (43, 44, 91, 92). UTI89 is apparently more dependent upon one or more of these activities when challenged with complement, whereas CFT073 can make do without CpxP. It is feasible that structural homologues of CpxP, such as Spy and ZraP (92–94), can substitute for CpxP under specific conditions in strains like CFT073. The Cpx system is best known for its effects on the expression of periplasmic chaperones and proteases in response to envelope stress, and the misregulation of these and other factors likely contribute to the myriad defects observed with the Δ*cpxP* and Δ*cpxRA* mutants in our assays.

ACKNOWLEDGMENTS

We thank Tamara Smith in the laboratory of Andrew Weyrich (University of Utah, Department of Human Genetics) for providing the human serum samples and Travis Wiles from our laboratory for initial assistance with the serum resistance assays. We are also grateful to Jacqueline Engel for help with characterization of the *yiiP* (*fieF*) mutants and Tracy Raivio for providing plasmids pNLP10-*lux* and pJW1-*cpxP-lux*.

This work was supported by grants AI095647, AI090369, and AI088086 from the National Institute of Allergy and Infectious Diseases. A.E.B. and R.R.K. were supported by NIH Microbial Pathogenesis Training Grant T32 AI055434, while J.P.N. was supported by NIH Genetics Training Grant T32-GM007464.

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APPENDIX D

ESCHERICHIA COLI O78 ISOLATED FROM SEPTICEMIC LAMBS SHOWS HIGH PATHOGENICITY IN A ZEBRAFISH MODEL

This work is currently under review for publication at
Veterinary Research

Kjelstrup CK, Barber AE, Norton JP, Mulvey MA & L-Abee-Lund TM. *Escherichia coli* O78 Isolated From Septicemic Lambs Shows High Pathogenicity in a Zebrafish Model.

Abstract

The pathogenicity of *Escherichia coli* O78 strain K46, originally isolated from an outbreak of septicemia in neonatal lambs, was investigated in zebrafish embryo and murine models of infection. Its biofilm potential, cellulose production, and the expression of type 1 pili and curli fimbriae were measured by *in vitro* assays. The strain was highly pathogenic in the zebrafish embryo model of infection, where it killed all embryos within 24 hours post inoculation at doses as low as 1000 colony forming units. Zebrafish embryos inoculated with similar doses of commensal *E. coli* strains showed no signs of disease, and cleared the bacteria within 24 hours. *E. coli* K46 colonized the murine gut at the same level as the uropathogenic *E. coli* (UPEC) reference strain CFT073 in CBA/J mice, but infected the murine bladder significantly less than CFT073. Type 1 pili were clearly expressed by *E. coli* K46, while curli fimbriae and cellulose production were weakly expressed. The ability to produce biofilm varied in different growth media, but overall *E. coli* K46 was a poorer biofilm producer compared to the reference strain *E. coli* UTI89.

Introduction

Escherichia coli is a Gram-negative, facultative anaerobic rod-shaped bacterium commonly found as part of the intestinal microbiota of warm-blooded vertebrates. Though often viewed as innert commensals, some strains of *E. coli* are able to cause disease. Pathogenic *E. coli* are genetically diverse and cause a variety of infections in animals and human beings. Pathogenic strains are

frequently categorized into diarrhogenic *E. coli* (DEC) or extraintestinal pathogenic *E. coli* (ExPEC). DEC and ExPEC are further subcategorized into specific pathogroups based on the site or mode of infection (Johnson and Russo, 2002). Among the diseases caused by ExPEC are septicemia, neonatal meningitis, and urinary tract infections (UTI). Despite causing disease outside the gut, such bacteria have been shown to colonize the gastrointestinal (GI) tract of the host, in some cases even more efficiently than commensal *E. coli* strains (Johnson and Russo, 2002). The GI tract is the main reservoir for pathogenic *E. coli* (Welch et al., 2002), and in septicemic disease, the GI tract together with the urogenital tract, are important portals of *E. coli* entry (Lefort et al., 2011).

ExPEC are genetically diverse and as such, there are no conserved virulence factors (VFs) or genetic characteristic that defines the ExPEC pathogroup or are essential for development of disease. Instead, septicemic bacteria possess a combination of VFs within the main groups of virulence traits (i.e., the adhesins, capsular synthesis, toxins, invasins and iron uptake systems). The set of VFs present in each septicemic strain enables the bacteria to defeat the host's defense mechanisms and cause an infection (Russo and Johnson, 2000). The lack of common VFs among septicemic strains and the numerous possible combinations of VFs make the recognition of septicemic strains and the assessment of their pathogenic potential challenging.

Genomic analysis can provide clues to a strain's virulence and demonstrate its similarity to other pathogenic isolates. However, to truly assess a

strain's pathogenic potential, it must be tested in a biological assay or animal model. Cell culture as well as invertebrate and vertebrate models have been developed to study septicemic disease and ExPEC virulence specifically. These models range from murine models aimed to differentiate pathogenic from nonpathogenic *E. coli* strains (Picard et al., 1999) to the unicellular amoeba model, *Dictyostelium discoideum,* measuring the level of bacterial pathogenicity (Adiba et al., 2010).

Zebrafish (*Danio rerio*) embryos have been shown to be a useful model organism for the study of host-pathogen interactions and the virulence potential of ExPEC strains (Wiles et al., 2009). During the early stages of development, the zebrafish immune system is composed solely of innate defenses, including phagocytes, NK cells, complement and toll-like receptors, and this corresponds to the immune system of neonatal lambs (Trede et al., 2004; Meeker and Trede, 2008; Meijer and Spaink, 2011).

E. coli O78 strain K46 was isolated from an outbreak of septicemia in neonatal lambs in Norway in 2008 (Kjelstrup et al., 2013). Previous genetic characterization could not explain the observed high virulence of this strain (Kjelstrup et al., 2013). The aim of this study was thus to investigate the virulence potential of *E. coli* K46 in *in vivo* virulence assays such as a zebrafish septicemia model, a mouse gut colonization model, and a murine urinary tract model, and in *in vitro* assays for the production of pili/fimbriae. In addition, the survival potential in the environment in a biofilm assay was examined.

Materials and Methods

Bacterial strains

The septicemic *E. coli* K46 was used in all the experiments in this study. Two uropathogenic *E. coli* strains, CFT073 and UTI89, and four nonpathogenic *E. coli* strains, designated Ctr 1- Ctr 4, were used as reference and control strains, respectively, for one or more of the experiments in this study (Table D.1).

Media and conditions

The minimal media M9 (6 g/l Na_2HPO_4, 3 g/l KH_2PO_4, 1 g/l NH_4Cl, 0.5 g/l NaCl, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 0.1% glucose, 0.0025% nicotinic acid and 16.5 μg/ml thiamine), YESCA (0.5 g/l yeast extract, 10 g/l Casamino Acids) and Luria-Bertani (LB) broth were used in one or more of the experiments. The cultures were grown overnight in an aerobic atmosphere at 37 °C shaking at 225 rpm if not otherwise specified.

In the zebrafish trial, inoculums were prepared by centrifugation of 1 ml of static, 24 hours M9 culture at 8000 rpm for 2 min, and the pellet was washed once with 1 ml sterile PBS (Hyclone) and resuspended in 275–550 μl PBS to obtain appropriate bacterial densities for microinjection.

In the mice gut colonization trial, 12 ml of a static overnight M9 culture was centrifuged at 8000 rpm for 10 min. The pellet was washed with 1 ml PBS, the centrifugation step repeated, and the pellet was resuspended in 500 μl sterile PBS. The number of colony forming units (CFU)/ml was measured after serial dilutions and plating to LB plates. For recovery of *E. coli* K46 and the reference

strain CFT073 in the mouse gut colonization study, tetracycline (5 mg/ml) and chloramphenicol (10 mg/ml), respectively, were added in the LB plates as selective agents.

In the mouse UTI trial, 20 ml of a static overnight M9 culture was centrifuged at 8000 rpm for 10 min at 20 °C and the pellet was resuspended in 8 ml sterile PBS and diluted 1:10. Serial dilutions and plating to LB plates were done to quantify the infection dose.

Zebrafish septicemia trials

The zebrafish septicemia model was used as in Wiles et al. (2009). Briefly, 48-54 hours old zebrafish embryos inoculated with approximately 10^3 CFU of *E. coli* into the bloodstream via the circulatory valley, resulting in rapid, systemic dispersal. Death was defined as the complete absence of heart rhythm and blood flow. To quantify bacterial burden during the course of infection, embryos from each infection group were collected at 6 and 12 hpi and individually homogenized with a mechanical PRO 250 homogenizer (PRO Scientific) in 0.5 ml PBS containing 0.5% Triton X-100. Homogenates were serially diluted and plated on LB agar, incubated overnight at 37 ºC and CFUs were counted.

To visually track the course of infection in real time, zebrafish embryos were also injected with 1-2×10^3 CFU of *E. coli* carrying pGEN-GFP (LVA) (REF Wiles et al., 2009) for constitutive expression of destabilized green fluorescent protein (GFP) protein (GFP-marked strains). At 12 hpi embryos were fixed by incubation overnight at 4 °C in PBS containing 4% paraformaldehyde in a light

proof container. Fixed embryos were washed in PBS containing 0.8% Triton X-100 in the dark for 4×20 minutes and then incubated for 10 min each in 30% and 50% glycerol before being transferred to a lightproof container and stored at 4 °C. Samples were imaged using an Olympus SZX10 stereomicroscope equipped with an Olympus DP72 camera.

Mouse gut colonization trials

Six-to-seven-week-old female CBA/J mice (Jackson Laboratory, USA) were anesthetized by inhalation of isoflurane gas and gavaged with 50 μl of approximately 3×10^9 CFU of either *E. coli* K46 (resistant to tetracycline) or CFT073-Clm^R (resistant to chloramphenicol). The two groups were kept in separate cages, and each mouse was marked individually. Fecal matter from each mouse was collected at the day of inoculation to ensure the absence of any tetracycline and chloramphenicol resistant bacteria in the commensal microbiota. Fecal samples were thereafter collected daily. Approximately 100 mg of feces/mouse/day was resuspended in 900 μl 0.7% NaCl. Serial dilutions were plated on LB agar with either chloramphenicol or tetracycline and incubated overnight at 37ºC for quantification of CFT073-Clm^R and *E. coli* K46, respectively. At 14 dpi the mice were anesthetized and euthanized by cervical dislocation, and the liver, kidney, bladder and intestines from the mice homogenized in 0.25% Triton X-100, serial diluted in 0.7% NaCl and plated on LB plates with tetracycline. The mice showed no overt signs of sickness throughout the trial.

Mouse UTI trials

Seven- to eight- week- old female CBA/J mice (Jackson Laboratory, USA) were anesthetized by isoflurane inhalation and slowly inoculated via transurethral catheterization as described by Wiles and coworkers (Wiles et al., 2013). Mice were inoculated with a 50 μl bacterial suspension of 1.6×10^6 - 2.4×10^5 CFU of *E. coli* K46. The mice were euthanized 3 days later by cervical dislocation under anesthesia, and the bladder and left kidney were homogenized separately in 1 ml of 0.025% Triton X-100. The suspensions were serially diluted, spread onto LB agar plates, and incubated for about 18 hours at 37 °C, and CFU/g tissue were calculated.

Expression of fimH, curli fiber and cellulose biosynthesis

The expression of fimH (adhesin of type 1 pili) was tested using a yeast agglutination assay. The strains were grown in static overnight LB cultures at 37 °C. 50 μl of PBS with 1% yeast (*Saccharomyces cerevisiae)* and 150 μl of overnight culture were pipetted onto a sterile microscope slide and mixed, and the ability to agglutinate was visually observed.

The expression of curli fiber was detected by growing the strains on Congo red plates (1% tryptone agar plates containing the dye Congo red) overnight at 30 ºC. Red pigment in the colonies was deemed as positive for curli fiber (Hernandes et al., 2011).

Since the expression of cellulose could reduce the production of biofilm (Zogaj et al., 2001), the bacterial strains were tested for their ability to synthesize

cellulose on LB agar with 0.02% (w/v) calcofluor white. Plates were incubated for 24 and 48 hours at 30 °C, and then observed under UV light (366 nm) for the presence of fluorescence and thereby positive for the synthesis of cellulose (Zogaj et al., 2001).

Growth curves and biofilm formation

E. coli strains were tested for their ability to form biofilm in three different media; LB, M9 and YESCA. Each overnight culture was diluted 1:100 in fresh medium, and four technical replicates of 100 μl of these dilutions were transferred to a flat-bottomed polystyrene 96-well microtiter plates with lids (NUNC). The plates were incubated at 30°C statically for 48 hours, the media was withdrawn, and the wells rinsed with water to remove any planktonic bacteria. 150 μl of 0.1% crystal violet solution was added to each well and incubated for 10 minutes at room temperature. The solution was removed and the wells were washed with water to remove any crystal violet unbound to biofilms before the microtiter plates were air dried without a lid for 30 min. 200 μl of solvent solution (DMSO) was added to solubilize stained biofilms and the plates were covered and incubated at room temperature, while shaking for 15 min. Absorbance was obtained using a SynergyHTTR/KC4 fluorimeter/luminometer (BioTek Instruments, Inc.) at 562 nm. Three biological replicates were made for each strain. The biofilm produced was measured by arbitrary units, where the mass of biofilm produced by the various strains was compared to the mass of biofilm produced by the reference strain UTI89.

To ensure that the observations were due to the strains' ability to form biofilm in each medium and not their ability to grow in these media, growth curves for the five strains in the three media were obtained using a Bioscreen C plate reader system (Growth Curves USA, Piscataway, NJ). The setup was parallel to the biofilm protocol regarding the preparation of the cultures, but 200 μl diluted culture was used instead of 100 μl in each well. The trays were inserted into the microplate reader (Bioscreen) for 24 hours with shaking, and bacterial growth was measured every 30 min at OD_{600}.

Ethics statement

Mice used in this study were handled in accordance with protocols approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Utah (Protocol number 10-02014), following US federal guidelines indicated by the Office of Laboratory Animal Welfare (OLAW) and described in the Guide for the Care and Use of Laboratory Animals, 8th Edition.

Zebrafish used in this study were handled in accordance with IACUC-approved protocols following standard procedures (www.zfin.org).

Results

E. coli K46 is highly virulent in the zebrafish bloodstream

E. coli K46 proliferated readily in the zebrafish embryos and approximately 2 and 3 magnitude higher levels of bacteria were retrieved at 6 and 12 hpi, respectively, compared to the time point of inoculation (Figure D.1B). Ctr 1 and

Ctr 2 did not reach higher levels in the embryos (Figure D.1B), and the bacteria were eliminated within 24 hpi (data not shown). All of the embryos infected with Ctr 1 and Ctr2 were still alive at 72 hpi (Figure D.1A) and no signs of obvious disease were observed in these fish. Conversely, all the embryos challenged with *E. coli* K46 died between12 and 18 hpi (Figure D.1A).

Infection with GFP-labeled *E. coli* K46 showed that bacteria were located throughout the vasculature at 12 hpi (Figure D.2), while the zebrafish embryos inoculated with the GFP-marked Ctr 1 and Ctr 2 were undetectable by eye at 12 hpi (data not shown). Together these results show that *E. coli* K46 is highly lethal in a zebrafish embryo model of infection and can replicate readily within the zebrafish bloodstream.

E. coli K46 stably colonizes the gastrointestinal tract of mice

Following oral gavage into the gastrointestinal tract, *E. coli* K46 colonized the gut of all mice at an average concentration of 10^6 CFU/g feces throughout the twelve days of experiment. This was similar to what was seen for ExPEC reference strain CFT073 (Figure D.3), indicating that *E. coli* K46 is an efficient colonizer of the mouse intestine.

E. coli K46 colonizes the bladder poorly

Following transurethral catheterization of K46 into the bladder, bacteria could not be detected at 3 dpi in half of the mice. In the remaining mice, 10^3-10^4 CFU/g tissue were isolated at this time point (Figure D.4). When bacterial titers in

the kidneys were examined, two of the mice with bacteria in the bladder also had 10^5-10^7 CFU/g bacteria present in the kidneys. A similar titer of bacteria was also found in the kidney of one mouse with no bacteria in the bladder (Figure D.4). The finding of bacteria in the kidneys indicates that *E. coli* K46 has the ability to invade host tissue, although it does not seem to be a primary pathogen of the urinary tract.

E. coli K46 expresses fimH, curli fiber
and produces cellulose

E. coli K46 and Ctr 1, Ctr 3 and Ctr 4 were positive for *fimH* expression as measured by yeast aggultination. *E. coli* K46 and *E. coli* Ctr 4 were weakly positive for the expression of curli fiber and cellulose production as determined by growth on Congo Red and Calcofluor White plates (Table D.2). These results suggest that *E. coli* K46 has all the requirements needed for biofilm production.

E. coli K46's ability to produce biofilm depends
on the trial conditions

In LB broth, both *E. coli* K46 and the control strains Ctr 1-Ctr 4 were poor biofilm producers (Figure D.5A). In YESCA media, Ctr 4 formed equal amount of biofilm as ExPEC reference strain UTI89, while the other strains produced approximately 50% less (Figure D.5C). In modified M9 minimal media, *E. coli* K46 and Ctr 1-Ctr 3, formed biofilm at the same or higher level than UTI89, while Ctr 4 formed approximately 20% less biofilm than UTI89 (Figure D.5B). The

growth characteristics of all *E. coli* strains tested were similar within each media, but differences in growth rate and final OD were observed between different media conditions (Figure D.5A-C). Together these data show that *E. coli* K46 produces biofilm most efficient under stringent conditions.

Discussion

Fewer virulence factors (VFs) were detected in *E. coli* K46 than in the control strains isolated from healthy lambs, but none of the detected VFs were sufficient to explain the observed lethality in the septicemic outbreak among the neonatal lambs (Kjelstrup et al., 2013). Despite this lack of specific VFs, we show that *E. coli* K46 is exceptionally virulent in the zebrafish model, as it killed all the infected embryos within 24 hpi, while none of the *E. coli* control strains caused any lethality. This is underscored by the zebrafish trial with the GFP marked *E. coli* K46, where the bacterial path from the infection site through the vasculature of the embryo is confirmed. The zebrafish trials correspond with the high virulence observed in the septicemic outbreak among the neonatal lambs, where all six lambs died after acute illness (Kjelstrup et al., 2013). The clearance of the *E. coli* control strains from the zebrafish embryos is in accordance with other studies where a virulent *E. coli* K12 (DH5α) was phagocytosed and eliminated within 5 hours after infection (Lavigne et al., 2012). While the zebrafish results do not explain the specific mechanisms behind the microbial pathogenesis, they do support our conviction that *E. coli* K46 is highly pathogenic. Our data also support the conclusion that bacterial factors and not only host factors, contributed

significantly to the lethality in the lambs.

The results obtained in the zebrafish trials further support the use of zebrafish as a suitable model organism for studying pathogenicity in *E. coli*. The zebrafish (*Danio rerio*) has a well-developed immune system, which is similar to the mammalian immune system (van der Sar et al., 2003). The model is also sensitive and hence applicable for examining pathogenicity and even closely related strains of ExPEC have been shown to display differences in virulence capability in the zebrafish model (Wiles et al., 2009).

The bacterial species in the GI tract of humans and sheep are different from those in mice, but the conditions and factors affecting bacterial competition within the mouse gut can to some extent be extrapolated to humans (Hao and Lee, 2004). Nevertheless, it was somewhat surprising that *E. coli* K46 colonized the murine gut at the same high levels as the reference strain CFT073, which is recognized to be a very efficient colonizer of the mouse intestine. In neonatal septicemia, the ewe may be the source of infection through contaminated fleece and udder after shedding pathogenic bacteria in the feces. The bacteria subsequently enter the lambs per os through suckling. From there the ingested bacteria likely colonize the infant gut and enter the bloodstream, resulting in septicemic disease. The portal of entry is not known in the *E. coli* K46 outbreak, but the strain's significant ability to colonize the murine gut indicate that the gastrointestinal tract as a possible entry route. If nothing else, the high and stable level of *E. coli* K46 in the mouse gut despite the absence of any selective

pressure shows that this strain has the essential prerequisites to outcompete an intact microbiota and colonize the gut. Whether *E. coli* K46 would establish itself as part of the normal murine gut flora permanently is not known, as the mice experiment was ended after 2 weeks duration when the level of *E. coli* K46 was still high.

Infection in the bladder and a subsequent ascending infection to the kidneys is a common infection route of septicemia in humans (Cusumano et al., 2010). *E. coli* K46 colonized the murine bladder poorly, which corresponds with the lack of pathological findings in the kidneys at autopsy of the mice (data not shown). This indicates that the urogenital tract is not a preferred entry route for the entrance of *E. coli* K46 into the bloodstream, at least not in mice. In two of three mice with *E. coli* in both the kidney and the bladder, the bacteria likely reached the kidney through an ascending infection. Bacterial reflux into the kidneys during transurethral catheterization is rare (Blango and Mulvey, 2010), but this might explain the retrieval of *E. coli* only in the kidney, and not in the bladder, in one of the mice.

E. coli K46 expressed curli fiber and the type 1 fimbriae *fimH*, in addition to possessing genes for other adhesins like *afaE VIII*, f17 fimbriae, *prfB, bmaE* and *lpfA* (Kjelstrup et al., 2013). The expression of different fimbriae and the level of their expression may give the strain a selective advantage during colonization, but may also play a role in the ability to form biofilm. Along with their role in the formation of secreted IgA-mediated biofilm within the gut, several groups have

reported that type 1 fimbriae (*fimH*) are critical for *E. coli* biofilm formation on abiotic surfaces (Pratt and Kolter, 1998; Moreira et al., 2003; Bollinger et al., 2006). Mutations in *fimH* have been reported to reduce *E. coli* attachment to polyvinyl chloride and other abiotic surfaces (Pratt and Kolter, 1998; Beloin et al., 2004). The expression of *fimH* has significance in the formation of biofilm in M9, while the expression of curli fiber is an important factor in biofilm formation in YESCA (Hadjifrangiskou et al., 2012). This is not supported by our study, where Ctr 2 (*fimH*-negative) formed biofilm at the same level as the reference strain in M9, while Ctr 4 (*fimH*-positive) did not. In YESCA, Ctr 4 formed biofilm on the same level as the reference strain, and at significantly higher level than *E. coli* K46, despite both strains being weakly positive for curli fiber. In addition, a direct antibiofilm role has been demonstrated for group II capsular polysaccharides (Naves et al., 2008), but since neither *E. coli* K46 nor Ctr 1-Ctr 4 contained genes for capsular synthesis this cannot explain the difference in biofilm production in the current study. Furthermore, one should keep in mind that this method of measuring biofilm only measures biomass and not cell viability in the biofilm (Crémet et al., 2013).

Cellulose is usually produced as an extracellular component for mechanical and chemical protection and the expression of cellulose can reduce biofilm production (Zogaj et al., 2001). However, the co-expression of cellulose and curli fiber can form a highly inert, hydrophobic extracellular matrix, called “bacterial wood” which surrounds the bacterial surface and functions as a survival

mechanism. The production of both cellulose and curli fiber in *E. coli* K46 could increase its persistence in a farm environment.

Conclusion

The zebrafish lethality model supports our belief that *E. coli* K46 is highly pathogenic, and that the lamb lethality seen in the septicemic outbreak was likely due to a collection of known and unknown bacterial virulence factors. The murine models show that *E. coli* K46 can stably colonize the intestinal microbiota and potentially provide a route of entry for infection. Virulence genes essential for the high pathogenicity are not identified, but bacterial knockout mutants used in zebrafish challenge could be an interesting approach in future studies.

Acknowledgements

This work was funded by the Research Council of Norway (project no. 190217/I10) and the Norwegian Meat and Poultry Research Centre (Animalia). Research in the Mulvey lab is supported by National Institutes of Health grant AI095647, NIH Microbial Pathogenesis Training Grant T32 AI055434 awarded to A.E.B., and NIH Training Grant T32 GM007464 to J.P.N.

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Table D.1. *E. coli* strains used in the study.

Strain	Source	Characteristics	Reference
E. coli wildtype strains			
K46	Septicemia, lamb	O78 Tetracycline and sulfonamide resistant Phylogenetic group C*	(Kjelstrup et al., 2013)
Ctr 1	Feces, healthy lamb	Phylogenetic group B2	(Kjelstrup et al., 2013)
Ctr 2	Feces, healthy lamb	Phylogenetic group B1	(Kjelstrup et al., 2013)
Ctr 3	Feces, healthy lamb	Phylogenetic group B2	(Kjelstrup et al., 2013)
Ctr 4	Feces, healthy lamb	Phylogenetic group B2	(Kjelstrup et al., 2013)
CFT073	Pyelonephritis, human	O6:K2:H1 Cytotoxic	(Mobley et al., 1990) (Welch et al., 2002)
UTI89	Cystitis-derived, human	O18:K1:H7	(Chen and Hung, 2006)
E. coli recombinant strains			
CFT073-Clm^R	CFT073	Chloramphenicol resistant	(Norton and Mulvey, 2012)
K46/pGEN-GFP	K46	Expresses destabilized green fluorescent protein (GFP)	This study
Ctr 1/pGEN-GFP	Ctr 1	Expresses destabilized GFP	This study
Ctr 2/pGEN-GFP	Ctr 2	Expresses destabilized GFP	This study

*Previously referred to as belonging to phylogenetic group A, but is now regrouped to Group C (Kjelstrup et al., 2013)

Table D.2. The expression of fimH and curli fiber and the production of cellulose in *E. coli* K46 and four control strains Ctr 1-Ctr 4.

Strains	fimH expression	Curli fiber	Cellulose synthesis
K46	+	+[a]	+[a]
Ctr 1	+	-	-
Ctr 2	-	-	-
Ctr 3	+	-	-
Ctr 4	+	+[a]	+[a]

[a] Weak reaction

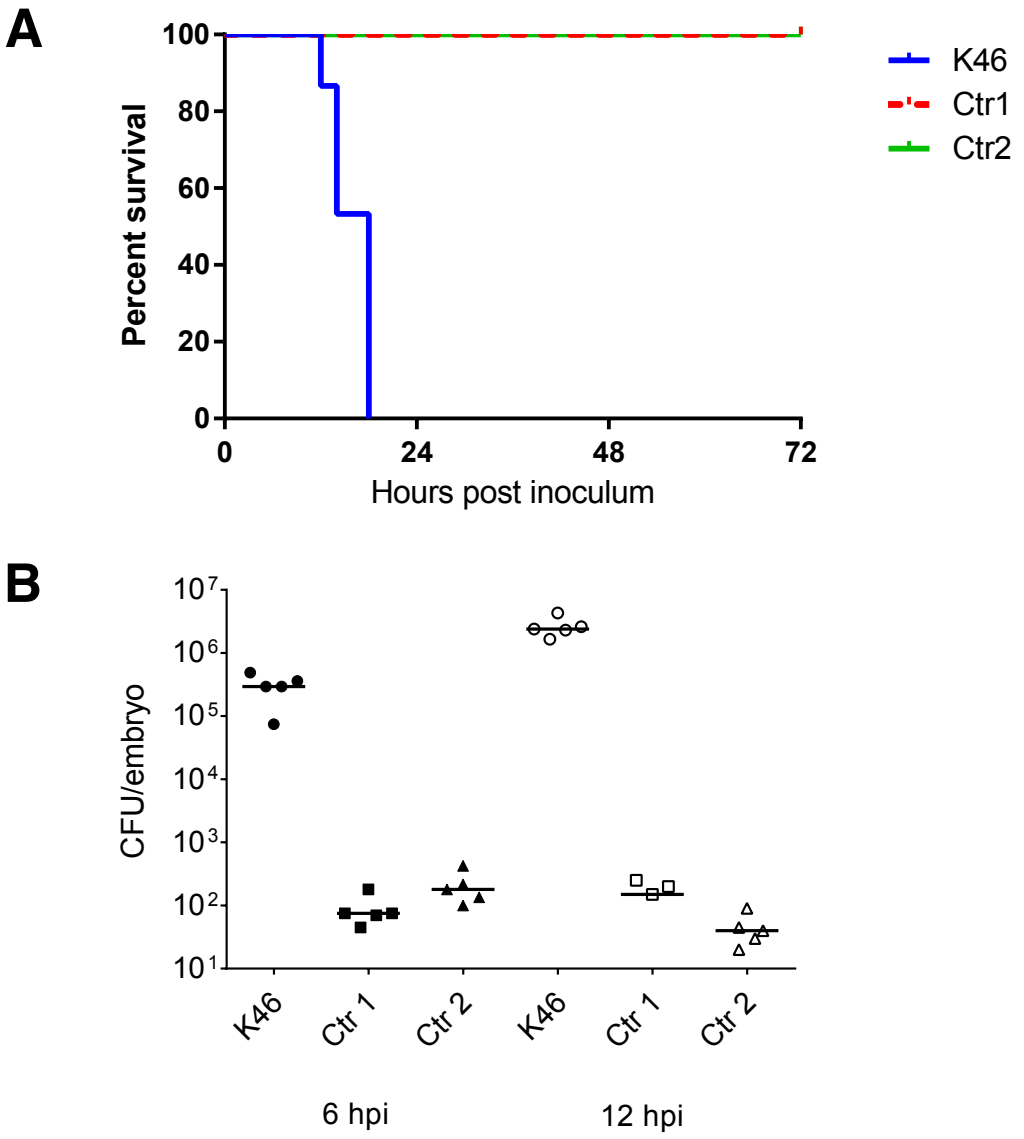

Figure D.1. Survival and bacterial titers in zebrafish embryos challenged with *E. coli* K46 and two control strains.

(**A**) Representative Kaplan-Meier survival plot of zebrafish embryos inoculated with 10^3 CFU of *E. coli* K46, Ctr 1 or Ctr 2, n = 18-20 embryos per group. **(B**) The level of bacteria retrieved from the inoculated zebrafish at 6 and 12 hours post inoculation (hpi). The horizontal bars indicate the median values for each group. Ctr 1 has two zero points at 12 hpi which are not graphed.

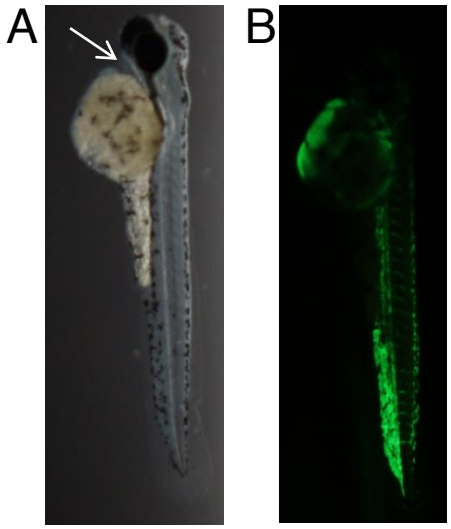

Figure D.2. Representative image of a zebrafish embryo inoculated with *E. coli* K46/pGEN-GFP (LVA) at 12 hpi.
(**A**) Brightfield image indicating the inoculation site in the circulatory valley (arrow). **(B**) The bacteria (GFP) are located throughout the vasculature of the embryo, but with minimal intrusion into the surrounding tissues.

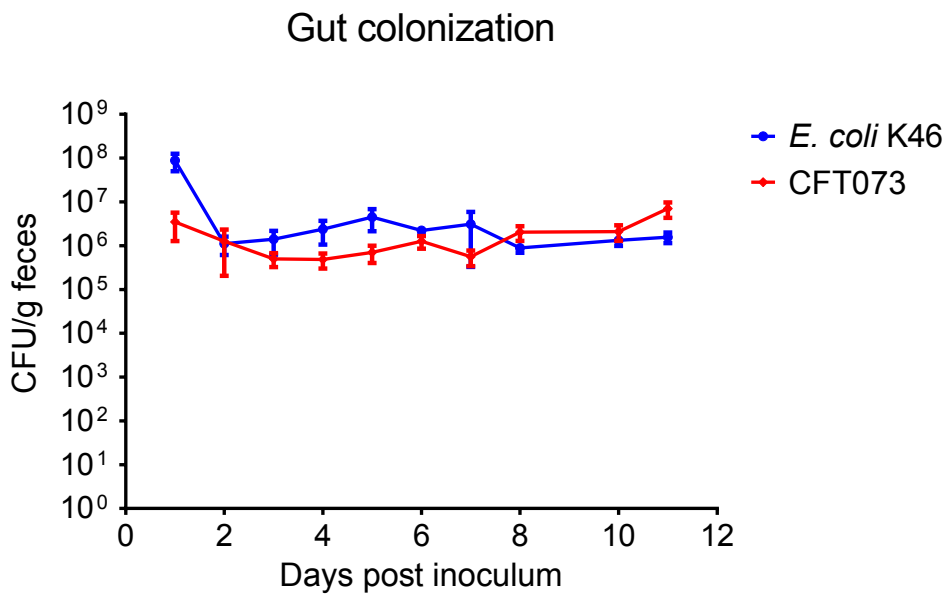

Figure D.3. Gastrointestinal tract colonization of adult female CBA/J mice gavaged with 1.5x10^8 CFU of *E. coli* K46 (tetracycline resistant) or the reference strain UPEC strain CFT073-ClmR (chloramphenicol resistant).
The colonization was assessed at each of the indicated time points by enumerating CFU per gram feces of the inoculated strains. Data represent CFU/g feces ± SEM (n = 5 mice).

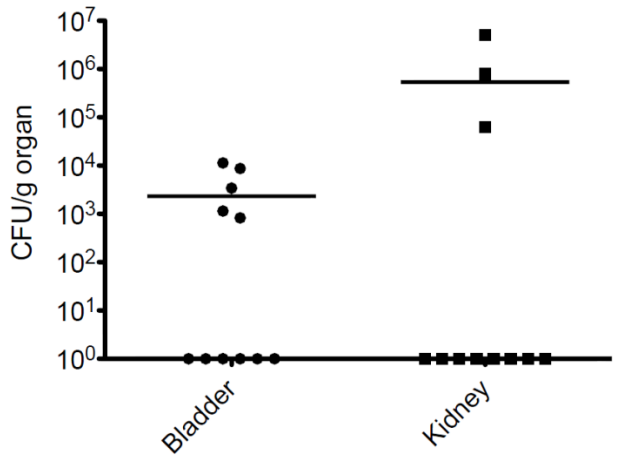

Figure D.4. Bacterial numbers of *E. coli* K46 present in the bladder and kidney of female CBA/J mice 3 days after transurethral catheterization of 10^5-10^6 CFU of *E. coli* K46.

The colonization was assessed at each of the indicated time points by enumerating CFU per gram feces of the inoculated strains. Data represent CFU/g feces ± SEM (n = 5 mice).

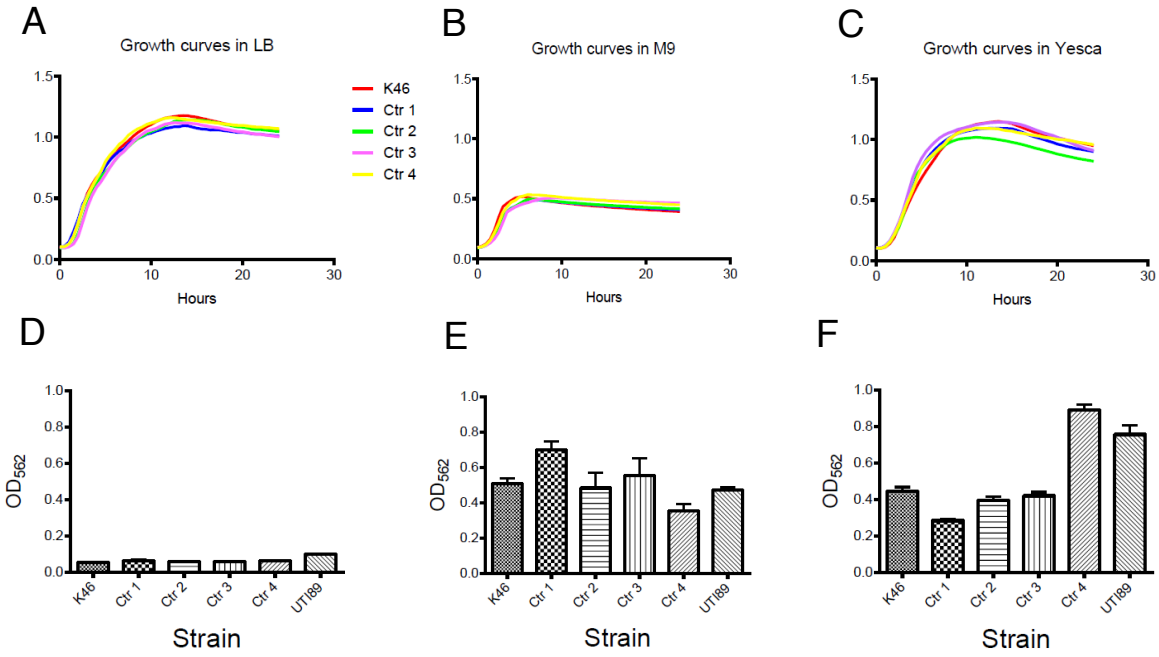

Figure D.5. Biofilm formation in *E. coli* K46 and four control strains (Ctr 1-Ctr 4) in LB, M9 minimal salts medium, and YESCA

(**A-C**) Representative growth curves in LB, M9, and in YESCA.

(**D-F**) Corresponding biofilm formation, measured relative to the reference strain UTI89. All biofilm data are the averages from three independent experiments performed in quadruplicate. Error bars indicate the standard deviation.